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(54) Title: B. BURGDORFERI POLYPEPTIDES EXPRESSED IN VIVO

(57) Abstract

Methods and compositions for the prevention, treatment and diagnosis of Lyme disease. Novel *B. burgdorferi* polypeptides, serotypic variants thereof, fragments thereof and derivatives thereof. Fusion proteins and multimeric proteins comprising same. Multicomponent vaccines comprising novel *B. burgdorferi* polypeptidesin addition to other immunogenic *B. burgdorferi* polypeptides. DNA sequences, recombinant DNA molecules and transformed host cells useful in the compositions and methods. Antibodies directed against the novel *B. burgdorferi* polypeptides, and diagnostic kits comprising the polypeptides or antibodies. A method for identifying bacterial genes that are selectively expressed *in vivo*.

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B. BURGDORFERI POLYPEPTIDES EXPRESSED IN VIVO

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TECHNICAL FIELD OF THE INVENTION

This invention relates to compositions and methods useful for the prevention, diagnosis and treatment of Lyme disease. More particularly, this invention relates to novel *B. burgdorferi* polypeptides which are able to elicit in a treated animal, the formation of an immune response. This invention also relates to novel *B. burgdorferi* polypeptides that are expressed during infection of a host but are not expressed by *B. burgdorferi* in in vitro culture.

This invention also relates to multicomponent vaccines comprising one or more of the novel *B. burgdorferi* polypeptides. Also within the scope of this invention are DNA sequences encoding the novel *B. burgdorferi* polypeptides, antibodies directed against the novel polypeptides and diagnostic kits comprising the antibodies or the polypeptides. Finally, this invention relates to novel methods for identifying bacterial genes that are selectively expressed in vivo.

BACKGROUND OF THE INVENTION

Lyme borreliosis is the most common vector-borne infection in the United States [S.W. Barthold, et al., "An Animal Model For Lyme Arthritis", Ann. N.Y. Acad. Sci., 539, pp. 264-73 (1988)] It has been reported in every continent except Antarctica. The clinical hallmark of Lyme disease is an early expanding skin lesion known as *erythema migrans*, which may be followed weeks to months later by neurologic, cardiac, and joint abnormalities.

The causative agent of Lyme disease is a spirochete known as *Borrelia burgdorferi*, transmitted primarily by *Ixodes* ticks of the *Ixodes ricinus* complex. *B. burgdorferi* has also been shown to be carried in other species of ticks and in mosquitoes and deer flies. But, it appears that only ticks of the *I. ricinus* complex are able to transmit the disease to humans

Lyme disease generally occurs in three stages. Stage one involves localized skin lesions (erythema migrans) from which the spirochete is cultured more readily than at any other time during infection [B.W. Berger et al., "Isolation And Characterization Of The Lyme Disease Spirochete From The Skin Of Patients With Erythema Chronicum Migrans", J. Am. Acad. Dermatol., 3, pp. 444-49 (1985)]. Flu-like or meningitis-like symptoms are common at this time. Stage two occurs within days or weeks, and involves spread of the spirochete through the patient's blood or lymph to many different sites in the body including the brain and joints. Varied symptoms of this disseminated infection occur in the skin, nervous system, and musculoskeletal system, although they are typically intermittent Stage three, or late infection, is defined as persistent infection, and can be severely disabling. Chronic arthritis, and syndromes of the central and peripheral nervous system appear during this stage, as a result of the ongoing infection and perhaps a resulting auto-immune disease [R. Martin et al., "*Borrelia burgdorferi*-Specific And Autoreactive T-Cell Lines From Cerebrospinal Fluid In Lyme Radiculomyelitis", Ann Neurol., 24, pp. 509-16 (1988)]

B. burgdorferi is much more difficult to culture from humans than from ticks. Therefore, at present, Lyme disease is diagnosed primarily by serology. The enzyme-linked immunosorbent assay (ELISA) is a frequently used method of detection. Typically, sonicated whole cultured spirochetes are used as the antigen in such assays to detect anti-*B. burgdorferi* antibodies formed in the serum of infected individuals [J.E. Craft et al., "The Antibody Response In Lyme Disease. Evaluation Of Diagnostic Tests", J. Infect. Dis., 149, pp 789-95 (1984)]. However, false negative and, more commonly, false positive results are associated with currently available tests.

At present, all stages of Lyme disease are treated with antibiotics. Treatment of early disease is usually effective. However, the cardiac, arthritic, and nervous system disorders associated with the later stages often do not respond to therapy [A.C. Steere, "Lyme Disease", New Eng. J. Med., 321, pp. 586-96 (1989)]. Early intervention, thus, is crucial for effective therapy. Accordingly, there exists an urgent need to identify immunogenic *B. burgdorferi* proteins that are expressed early in infection.

Like *Treponema pallidum*, which causes syphilis, and leptospirae, which cause an infectious jaundice, *Borrelia* belong to the eubacterial phylum of spirochetes [A.G. Barbour and S.F. Hayes, "Biology Of Borrelia Species", Microbiol. Rev., 50, pp. 381-400 (1986)]. *Borrelia burgdorferi* have a protoplasmic cylinder that is surrounded by a cell membrane, then by flagella, and then by an outer membrane.

The *B. burgdorferi* outer surface proteins identified to date are believed to be lipoproteins, as demonstrated by labelling with [^{3}H]palmitate [M.E. Brandt et al., "Immunogenic Integral membrane Proteins of *Borrelia burgdorferi* Are Lipoproteins", Infect. Immun., 58, pp. 983-91 (1990)]. The two major outer surface proteins are the 31 kDa outer-surface protein A (OspA) and the 34 kDa outer surface protein B (OspB). Both proteins have been shown to vary from

different isolates or from different passages of the same isolate as determined by their molecular weights and reactivity with monoclonal antibodies. OspC is a 22 kDa membrane lipoprotein previously identified as pC [R. Fuchs et al., "Molecular Analysis and Expression of a *Borrelia burgdorferi* Gene Encoding a 22 kDa Protein (pC) in *Escherichia coli*", Mol. Microbiol., 6, pp. 503-09 (1992)]. OspD is said to be preferentially expressed by low-passage, virulent strains of *B. burgdorferi* B31 [S.J. Norris et al., "Low-Passage-Associated Proteins of *Borrelia burgdorferi* B31: Characterization and Molecular Cloning of OspD, A Surfaced-Exposed, Plasmid-Encoded Lipoprotein", Infect. Immun., 60, pp. 4662-4672 (1992)]. OspE, a 19 kD protein, is expressed early in infection while OspF, a 26 kD protein, is expressed at a later stage [T.T. Lam et al., "Outer Surface Proteins E and F Of *Borrelia burgdorferi*, the Agent of Lyme Disease," Infect. Immun., 62, pp. 290-298 (1994)].

Non-Osp *B. burgdorferi* proteins identified to date include the 41 kDa flagellin protein, which is known to contain regions of homology with other bacterial flagellins [G.S. Gassman et al., "Analysis of the *Borrelia burgdorferi* GeHo *fla* Gene and Antigenic Characterization of Its Gene Product", J. Bacteriol., 173, pp. 1452-59 (1991)] and a 93 kDa protein said to be localized to the periplasmic space [D.J. Volkman et al., "Characterization of an Immunoreactive 93 kDa Core Protein of *Borrelia burgdorferi* With a Human IgG Monoclonal Antibody", J. Immun., 146, pp. 3177-82 (1991)].

B. burgdorferi is known to alter the antigens on its outer surface during different stages of its life cycle. For example, OspC is not expressed by spirochetes within unfed ticks. However, it is synthesized following engorgement and the introduction of a blood meal into the lumen of the tick's midgut. In contrast, OspA is a prominent surface antigen on spirochetes within the midguts of resting ticks. As spirochetes migrate from the midgut to the salivary gland during the tick feeding, OspA expression decreases. The downregulation of OspA within ticks allows spirochetes to survive in the presence of an OspA antibody response,

suggesting that selective antigen expression may be a mechanism by which *B. burgdorferi* evade immune destruction.

It is known that the expression of other bacterial pathogen gene products is induced by environmental signals [J J. Mekalanos, "Environmental Signal Controlling Expression of Virulence Determinants In Bacteria," J. Bacteriol., 174, pp 1-7 (1992)] A similar induction of gene expression may occur in the infected host where specific external signals are present. Thus, to understand the mechanism of pathogenesis, it is important to identify genes that are expressed in the host but not in in vitro culture and then to study the function of the gene product

A genetic system using *Salmonella typhimurium* has been developed to identify bacterial genes induced in vivo [M. J. Mahan et al, "Selection of Bacterial Virulence Genes That Are Specifically Induced in Host Tissues," Science, 259, pp 686-688 (1993)]. However, this system may not be applied to pathogenic organisms for which a gene transfer system and a well-defined auxotroph are not available. Such systems are unavailable in *B. burgdorferi*. Because effective treatment and prevention of Lyme disease requires an understanding of the mechanisms that allow *B. burgdorferi* to evade host defenses, cause disease and survive within the host, there is an urgent need for a method to identify *B. burgdorferi* genes that are selectively expressed in vivo.

The humoral response to *B. burgdorferi* antigens that are expressed only within the vertebrate host may aid in the serologic diagnosis of Lyme disease. Such proteins are not present on spirochetes cultured in Barbour-Stoenner-Kelly (BSK) II medium. Selective in vivo expression of some *B. burgdorferi* proteins may be one reason that current diagnostic tests for Lyme disease, based on whole-cell lysates of cultured *B. burgdorferi*, are unreliable. Such tests cannot detect antibodies directed toward the in vivo expressed antigens. Accordingly, there also

exists a need to identify *B. burgdorferi* proteins that provide more reliable diagnostic tests for Lyme disease.

Recently, immunization of mice with recombinant OspA has been shown to be effective to confer long-lasting protection against subsequent infection with *B. burgdorferi* [E. Fikrig et al., "Long-Term Protection of Mice from Lyme Disease by Vaccination with OspA", Infec. Immun., 60, pp. 773-77 (1992)]. However, protection by the OspA immunogens used to date appears to be somewhat strain specific, probably due to the heterogeneity of the OspA gene among different *B. burgdorferi* isolates. For example, immunization with OspA from *B. burgdorferi* strain N40 confers protection against subsequent infection with strains N40, B31 and CD16, but not against strain 25015 [E. Fikrig et al., "*Borrelia burgdorferi* Strain 25015: Characterization of Outer Surface Protein A and Vaccination Against Infection", J. Immun., 148, pp. 2256-60 (1992)].

Immunization with OspB has also been shown to confer protection against Lyme disease but not to the same extent as that conferred by OspA [E. Fikrig et al., "Roles of OspA, OspB, and Flagellin in Protective Immunity to Lyme Borreliosis in Laboratory Mice", Infec. Immun., 60, pp. 657-61 (1992)]. Moreover, some *B. burgdorferi* are apparently able to escape destruction in OspB-immunized mice via a mutation in the OspB gene which results in expression of a truncated OspB protein [E. Fikrig et al., "Evasion of Protective Immunity by *Borrelia burgdorferi* by Truncation of Outer Surface Protein B", Proc. Natl. Acad. Sci., 90, pp. 4092-96 (1993)]. OspC has also been shown to have protective effects in a gerbil model of *B. burgdorferi* infection. However, the protection afforded by immunization with this protein appears to be only partial [V. Preac-Mursic et al., "Active Immunization with pC Protein of *Borrelia burgdorferi* Protects Gerbils against *B. burgdorferi* Infection", Infection, 20, pp. 342-48 (1992)].

Immunization with OspF has also been shown to confer partial protection against infection [T. K. Nguyen et al., "Partial Destruction of *Borrelia*

burgdorferi Within Ticks That Engorged On OspE- Or OspF-Immunized Mice," Infect. Immun., 62, pp. 2079-2084 (1994)]. Both anti-OspE and anti-OspF antibodies have been shown to reduce the number of spirochetes in ticks [T.K Nguyen et al., supra].

As prevention of tick infestation is imperfect, and Lyme disease may be missed or misdiagnosed when it does appear, there exists a continuing urgent need for the determination of additional antigens of *B. burgdorferi* and related proteins which are able to elicit a protective immune response and which may be useful in a broad-spectrum vaccine. In addition, identification of additional *B. burgdorferi* antigens may enable the development of more reliable diagnostic reagents which are useful in various stages of Lyme borreliosis.

DISCLOSURE OF THE INVENTION

The present invention provides novel *B. burgdorferi* polypeptides which are substantially free of a *B. burgdorferi* spirochete or fragments thereof and, thus, are useful in compositions and methods for the diagnosis, treatment and prevention of *B. burgdorferi* infection and Lyme disease. In one embodiment, this invention provides P21 polypeptides and compositions and methods comprising those polypeptides.

In another embodiment, this invention provides K2 polypeptides and compositions and methods comprising those polypeptides.

In another embodiment, this invention provides P35 polypeptides and compositions and methods comprising those polypeptides.

In another embodiment, this invention provides P37 polypeptides and compositions and methods comprising those polypeptides.

In another embodiment, this invention provides M30 polypeptides and compositions and methods comprising those polypeptides.

In another embodiment, this invention provides V3 polypeptides and compositions and methods comprising those polypeptides.

In another embodiment, this invention provides J1 polypeptides compositions and methods comprising those polypeptides.

In another embodiment, this invention provides J2 polypeptides compositions and methods comprising those polypeptides.

The preferred polypeptides of each of the aforementioned embodiments are selectively expressed in vivo.

Also preferred are compositions and methods of each of the aforementioned embodiments are characterized by novel *B. burgdorferi* polypeptides which elicit in treated animals the formation of an immune response

In another embodiment, this invention provides a multicomponent vaccine comprising one or more novel *B. burgdorferi* polypeptides of this invention in addition to one or more other immunogenic *B. burgdorferi* polypeptides. Such a vaccine is effective to confer broad protection against *B. burgdorferi* infection.

In yet another embodiment, this invention provides antibodies directed against the novel *B. burgdorferi* polypeptides of this invention, and compositions and methods comprising those antibodies.

In another embodiment, this invention provides diagnostic means and methods characterized by one or more of the novel *B. burgdorferi* polypeptides, or antibodies directed against those polypeptides. These means and methods are useful for the detection of Lyme disease and *B. burgdorferi* infection. They are also useful in following the course of treatment against such infection In patients previously inoculated with the vaccines of this invention, the detection means and methods disclosed herein are also useful for determining if booster inoculations are appropriate.

In yet another embodiment, this invention provides methods for the identification and isolation of additional *B. burgdorferi* polypeptides, as well as compositions and methods comprising such polypeptides.

In yet another embodiment, this invention provides methods for identifying bacterial genes encoding an antigenic protein which is expressed during infection of a host but is not expressed during in vitro culture of the bacteria.

Finally, this invention provides DNA sequences that code for the novel *B. burgdorferi* polypeptides of this invention, recombinant DNA molecules that are characterized by those DNA sequences, unicellular hosts transformed with those DNA sequences and molecules, and methods of using those sequences, molecules and hosts to produce the novel *B. burgdorferi* polypeptides and multicomponent vaccines of this invention. DNA sequences of this invention are also advantageously used in methods and means for the diagnosis of Lyme disease and *B. burgdorferi* infection.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the DNA and amino acid sequences of the P21 polypeptide of *B. burgdorferi* strain N40

Figure 2 depicts the DNA and amino acid sequences of the P35 polypeptide of *B. burgdorferi* strain N40

Figure 3 depicts the DNA and amino acid sequences of the P37 polypeptide of *B. burgdorferi* strain N40

Figure 4 depicts the DNA and amino acid sequences of the M30 polypeptide of *B. burgdorferi* strain N40

Figure 5 depicts the DNA and amino acid sequences of the V3 polypeptide of *B. burgdorferi* strain N40.

Figure 6 depicts the hydrophilicity profiles of P35 and P37.

Figure 7 depicts a comparison of the amino acid sequences of P21 and *B. burgdorferi* strain N40 OspE.

Figure 8 depicts a comparison of the control regions of transcription and translation among the DNA sequences encoding P21 and K2 and the DNA sequences of other known *B. burgdorferi* outer surface proteins.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to novel *B. burgdorferi* polypeptides, the DNA sequences which encode them, antibodies directed against those polypeptides, compositions comprising the polypeptides or antibodies, and methods for the detection, treatment and prevention of Lyme disease.

More specifically, in one embodiment, this invention relates to P21 polypeptides and compositions and methods comprising those polypeptides.

In another embodiment, this invention relates to K2 polypeptides and compositions and methods comprising those polypeptides.

In another embodiment, this invention relates to P35 polypeptides and compositions and methods comprising those polypeptides.

In another embodiment, this invention relates to P37 polypeptides and compositions and methods comprising those polypeptides.

In another embodiment, this invention relates to M30 polypeptides and compositions and methods comprising those polypeptides.

In another embodiment, this invention relates to V3 polypeptides and compositions and methods comprising those polypeptides.

In another embodiment, this invention relates to J1 and compositions and methods comprising those polypeptides.

In another embodiment, this invention relates to J2 and compositions and methods comprising those polypeptides.

The preferred polypeptides, compositions and methods of each of the aforementioned embodiments are characterized by novel *B. burgdorferi* polypeptides that are immunogenic *B. burgdorferi* polypeptides.

In another embodiment, this invention relates to a multicomponent vaccine against Lyme disease comprising one or more of the novel *B. burgdorferi* polypeptides of this invention in addition to other immunogenic *B. burgdorferi* polypeptides. Such vaccine is useful to protect against infection by a broad spectrum of *B. burgdorferi* organisms.

All of the novel *B. burgdorferi* polypeptides provided by this invention, and the DNA sequences encoding them, may be produced substantially free of *B. burgdorferi* spirochete or fragments thereof, and thus may be used in a variety of applications without the risk of unintentional infection or contamination with undesired *B. burgdorferi* components. Accordingly, the novel *B. burgdorferi* polypeptides of this invention are particularly advantageous in compositions and methods for the diagnosis and prevention of *B. burgdorferi* infection.

In another embodiment, this invention relates to compositions and methods comprising antibodies directed against the novel *B. burgdorferi* polypeptides of this invention. Such antibodies may be used in a variety of applications, including to detect the presence of *B. burgdorferi*, to screen for expression of novel *B. burgdorferi* polypeptides, to purify novel *B. burgdorferi* polypeptides, to block or bind to the novel *B. burgdorferi* polypeptides, to direct molecules to the surface of *B. burgdorferi*, to prevent or lessen the severity, for some period of time, of *B. burgdorferi* infection, and to decrease the level of *B. burgdorferi* spirochetes in ticks.

In still another embodiment, this invention relates to diagnostic means and methods characterized by the novel *B. burgdorferi* polypeptides disclosed herein or antibodies directed against those polypeptides.

In yet another embodiment, this invention relates to methods for identifying bacterial genes that are selectively expressed in vivo.

In order to further define this invention, the following terms and definitions are herein provided

As used herein, an "immunogenic *B. burgdorferi* polypeptide" is any *B. burgdorferi* polypeptide that, when administered to an animal, is capable of eliciting an immune response

Immunogenic *B. burgdorferi* polypeptides are intended to include not only the novel *B. burgdorferi* polypeptides of this invention but also the OspA and OspB polypeptides disclosed in PCT patent application WO 92/00055; the OspC protein as described in R. Fuchs et al., *supra*, the OspE and OspF polypeptides disclosed in PCT patent application WO 95/04145; other *B. burgdorferi* proteins; and fragments, serotypic variants and derivatives of any of the above. In particular, immunogenic *B. burgdorferi* polypeptides are intended to include additional *B. burgdorferi* polypeptides which are identified according to the methods disclosed herein.

As used herein, a polypeptide which is "substantially free of a *B. burgdorferi* spirochete or fragments thereof" is a polypeptide that, when introduced into modified Barbour-Stoener-Kelly (BSK-II) medium and cultured at 37°C for 7 days, fails to produce any *B. burgdorferi* spirochetes detectable by dark field microscopy or a polypeptide that is detectable as a single band on an immunoblot probed with polyclonal anti-*B. burgdorferi* anti-serum.

As used herein, a *B. burgdorferi* polypeptide that is "selectively expressed in vivo" is a polypeptide encoded by a DNA sequence that corresponds to a *B. burgdorferi* gene that is expressed during infection of a host but is not expressed during in vitro culture of said *B. burgdorferi*. A DNA sequence that "corresponds to a *B. burgdorferi* gene" is a DNA sequence that encodes a

polypeptide that is the same as, a fragment of or a derivative of a naturally occurring *B. burgdorferi* polypeptide

As used herein, a "P21 polypeptide" denotes a polypeptide which is selected from the group consisting of

(a) a P21 polypeptide consisting of amino acids 1-182 of SEQ ID NO: 2,

(b) fragments comprising at least 15 amino acids taken as a block from the P21 polypeptide of (a); and

(c) a polypeptide that is selectively expressed in vivo and that:

(1) is a derivative of a P21 polypeptide of (a), said derivative being at least 80% identical in amino acid sequence to the corresponding polypeptide of (a);

(2) polypeptides that are immunologically reactive with antibodies generated by infection of a mammalian host with *B. burgdorferi*, which antibodies are immunologically reactive with a P21 polypeptide of (a);

(3) polypeptides that are capable of eliciting antibodies that are immunologically reactive with *B. burgdorferi* and the P21 polypeptide of (a) and

(4) polypeptides that are immunologically reactive with antibodies elicited by immunization with the P21 polypeptide of (a).

As used herein, a "K2 polypeptide" denotes a polypeptide which is selected from the group consisting of

(a) a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 3,

(b) derivatives of the polypeptide of (a), said derivative comprising a polypeptide having a block of amino acids at least 80% identical in sequence to SEQ ID NO: 3; and

(c) a polypeptide that is selectively expressed in vivo and that:

(1) is a derivative of a polyeptide of (a), said derivative being at least 80% identical in amino acid sequence to the corresponding polypeptide of (a),

(2) polypeptides that are immunologically reactive with antibodies generated by infection of a mammalian host with *B. burgdorferi*, which antibodies are immunologically reactive with a polypeptide of (a),

(3) polypeptides that are capable of eliciting antibodies that are immunologically reactive with *B. burgdorferi* and the polypeptide of (a), and

(4) polypeptides that are immunologically reactive with antibodies elicited by immunization with the polypeptide of (a)

As used herein, a "P35 polypeptide" denotes a polypeptide which is selected from the group consisting of

(a) a P35 protein comprising the amino acid sequence set forth in SEQ ID NO: 5 and serotypic variants thereof;

(b) fragments comprising at least 8 amino acids taken as a block from the P35 polypeptide of (a);

(c) derivatives of the P35 polypeptide of (a) or (b), said derivatives being at least 80% identical in amino acid sequence to the corresponding polypeptide of (a) or (b);

(d) polypeptides that are immunologically reactive with antibodies generated by infection of a mammalian host with *B. burgdorferi*, which antibodies are immunologically reactive with a P35 polypeptide of (a) or (b) or (c);

(e) polypeptides that are capable of eliciting antibodies that are immunologically reactive with *B. burgdorferi* and the P35 polypeptide of (a) or (b) or (c); and

(f) polypeptides that are immunologically reactive with antibodies elicited by immunization with the P35 polypeptide of (a) or (b) or (c)

As used herein, a "P37 polypeptide" denotes a polypeptide which is selected from the group consisting of

(a) a P37 protein having the amino acid sequence of SEQ ID NO: 7 and serotypic variants thereof;

(b) fragments comprising at least 8 amino acids taken as a block from the P37 polypeptide of (a);

(c) derivatives of the P37 polypeptide of (a) or (b), said derivatives being at least 80% identical in amino acid sequence to the corresponding polypeptide of (a) or (b);

(d) polypeptides that are immunologically reactive with antibodies generated by infection of a mammalian host with *B. burgdorferi*, which antibodies are immunologically reactive with a P37 polypeptide of (a) or (b) or (c);

(e) polypeptides that are capable of eliciting antibodies that are immunologically reactive with *B. burgdorferi* and the P37 polypeptide of (a) or (b) or (c); and

(f) polypeptides that are immunologically reactive with antibodies elicited by immunization with the P35 polypeptide of (a) or (b) or (c).

As used herein, a "M30 polypeptide" denotes a polypeptide which is selected from the group consisting of:

(a) a M30 polypeptide having the amino acid sequence of SEQ ID NO: 9 and serotypic variants thereof;

(b) fragments comprising at least 8 amino acids taken as a block from the M30 polypeptide of (a);

(c) derivatives of the M30 polypeptide of (a) or (b), said derivatives being at least 80% identical in amino acid sequence to the corresponding polypeptide of (a) or (b);

(d) polypeptides that are immunologically reactive with antibodies generated by infection of a mammalian host with *B. burgdorferi*, which antibodies are immunologically reactive with a M30 polypeptide of (a) or (b) or (c);

(e) polypeptides that are capable of eliciting antibodies that are immunologically reactive with *B. burgdorferi* and the M30 polypeptide of (a) or (b) or (c); and

(f) polypeptides that are immunologically reactive with antibodies elicited by immunization with the M30 polypeptide of (a) or (b) or (c)

As used herein, a "V3 polypeptide"denotes a polypeptide which is selected from the group consisting of

(a) a V3 protein having an amino acid sequence encoded by SEQ ID NO: 10 and serotypic variants thereof;

(b) fragments comprising at least 8 amino acids taken as a block from the polypeptide of (a);

(c) derivatives of the polypeptide of (a) or (b), said derivatives being at least 80% identical in amino acid sequence to the corresponding polypeptide of (a) or (b),

(d) polypeptides that are immunologically reactive with antibodies generated by infection of a mammalian host with *B. burgdorferi*, which antibodies are immunologically reactive with a polypeptide of (a) or (b) or (c);

(e) polypeptides that are capable of eliciting antibodies that are immunologically reactive with *B. burgdorferi* and the polypeptide of (a) or (b) or (c), and

(f) polypeptides that are immunologically reactive with antibodies elicited by immunization with the polypeptide of (a) or (b) or (c)

As used herein, a "V3 polypeptide" is intended to include a *B. burgdorferi* polypeptide encoded in whole or in part by the *B. burgdorferi* DNA sequence contained in ATCC deposit No. __, which cross-hybridizes to the DNA sequence of SEQ ID NO: 10

As used herein, a "J1 polypeptide" denotes a polypeptide which is selected from the group consisting of

(a) a polypeptide encoded in whole or in part by the *B. burgdorferi* DNA sequence contained within ATCC deposit No (2) and serotypic variants thereof,

(b) fragments comprising at least 8 amino acids taken as a block from the polypeptide of (a),

(c) derivatives of the polypeptide of (a) or (b), said derivatives being at least 80% identical in amino acid sequence to the corresponding polypeptide of (a) or (b);

(d) polypeptides that are immunologically reactive with antibodies generated by infection of a mammalian host with *B. burgdorferi*, which antibodies are immunologically reactive with a polypeptide of (a) or (b) or (c);

(e) polypeptides that are capable of eliciting antibodies that are immunologically reactive with *B. burgdorferi* and the polypeptide of (a) or (b) or (c), and

(f) polypeptides that are immunologicaliy reactive with antibodies elicited by immunization with the polypeptide of (a) or (b) or (c).

As used herein, a "J1 polypeptide" is intended to include a *B. burgdorferi* polypeptide encoded in whole or in part by the *B. burgdorferi* DNA sequence contained within ATCC deposit No (2A), which cross-hybridizes to the *B. burgdorferi* DNA sequence contained within ATCC deposit No. __

As used herein, a "J2 polypeptide" denotes a polypeptide which is selected from the group consisting of:

(a) a polypeptide encoded in whole or in part by the *B. burgdorferi* DNA sequence contained within ATCC deposit No (3) and serotypic variants thereof;

(b) fragments comprising at least 8 amino acids taken as a block from the polypeptide of (a);

(c) derivatives of the polypeptide of (a) or (b), said derivatives being at least 80% identical in amino acid sequence to the corresponding polypeptide of (a) or (b),

(d) polypeptides that are immunologically reactive with antibodies generated by infection of a mammalian host with *B. burgdorferi*, which antibodies are immunologically reactive with a polypeptide of (a) or (b) or (c);

(e) polypeptides that are capable of eliciting antibodies that are immunologically reactive with *B. burgdorferi* and the polypeptide of (a) or (b) or (c); and

(f) polypeptides that are immunologically reactive with antibodies elicited by immunization with the polypeptide of (a) or (b) or (c).

As used herein, a "J2 polypeptide" is intended to include a *B. burgdorferi* polypeptide encoded in whole or in part by the *B. burgdorferi* DNA sequence contained within ATCC deposit Nos (3A and 3B), which cross-hybridize to the *B. burgdorferi* DNA sequence contained within ATCC deposit No. (3)

As used herein, a "novel *B. burgdorferi* polypeptide" is a P21 polypeptide, a K2 polypeptide, a P35 polypeptide, a P37 polypeptide, an M30 polypeptide, a V3 polypeptide, a J1 polypeptide or a J2 polypeptide.

As used herein, a "serotypic variant" of a novel *B. burgdorferi* polypeptide according to this invention is any naturally occurring polypeptide which may be encoded in whole or in part, by a DNA sequence which hybridizes, at 20-27°C below Tm, to the DNA sequence encoding the novel *B. burgdorferi* polypeptide. One of skill in the art will understand that serotypic variants of a novel *B. burgdorferi* polypeptide according to this invention include polypeptides encoded by DNA sequences of which any portion may be amplified by using the polymerase chain reaction and oligonucleotide primers derived from any portion of the DNA sequence encoding the novel *B. burgdorferi* polypeptide.

As used herein, a "derivative" of a novel *B. burgdorferi* polypeptide according to his invention is a novel *B. burgdorferi* polypeptide in which one or more physical, chemical, or biological properties has been altered. Such modifications include, but are not limited to amino acid substitutions,

modifications, additions or deletions, alterations in the pattern of lipidation, glycosylation or phosphorylation, reactions of free amino, carboxyl, or hydroxyl side groups of the amino acid residues present in the polypeptide with other organic and non-organic molecules, and other modifications, any of which may result in changes in primary, secondary or tertiary structure.

As used herein, a "protective antibody" is an antibody that confers protection, for some period of time, against any one of the physiological disorders associated with *B. burgdorferi* infection.

As used herein, a "protective *B. burgdorferi* polypeptide" is a polypeptide that comprises a protective epitope.

As used herein, a "protective epitope" is (1) an epitope which is recognized by a protective antibody, and/or (2) an epitope which, when used to immunize an animal, elicits an immune response sufficient to prevent or lessen the severity for some period of time, of *B. burgdorferi* infection.

Preventing or lessening the severity of infection may be evidenced by a change in the physiological manifestations of erythema migrans, arthritis, carditis, neurological disorders, and other Lyme disease related disorders. It may be evidenced by a decrease in the level of spirochetes in the treated animal. And, it may also be evidenced by a decrease in the level of spirochetes in infected ticks feeding on treated animals. A protective epitope may comprise a T cell epitope, a B cell epitope, or combinations thereof.

As used herein, a "T cell epitope" is an epitope which, when presented to T cells by antigen presenting cells, results in a T cell response such as clonal expansion or expression of lymphokines or other immunostimulatory molecules. A T cell epitope may also be an epitope recognized by cytotoxic T cells that may affect intracellular *B. burgdorferi* infection. A strong T cell epitope is a T cell epitope which elicits a strong T cell response.

As used herein, a "B cell epitope" is the simplest spatial conformation of an antigen which reacts with a specific antibody.

As used herein, a "therapeutically effective amount" of a polypeptide or of an antibody is the amount that, when administered to an animal, elicits an immune response that is effective to prevent or lessen the severity, for some period of time, of *B. burgdorferi* infection.

As used herein, an "antibody directed against a novel *B. burgdorferi* polypeptide" (also referred to as "an antibody of this invention") is an antibody directed against a P21 polypeptide, a K2 polypeptide, a P35 polypeptide, a P37 polypeptide, an M30 polypeptide, a V3 polypeptide, a J1 polypeptide or a J2 polypeptide. It should be understood that an antibody directed against a novel *B. burgdorferi* polypeptide may also be a protective antibody.

An antibody directed against a novel *B. burgdorferi* polypeptide may be an intact immunoglobulin molecule or a portion of an immunoglobulin molecule that contains an intact antigen binding site, including those portions known in the art as F(v), Fab, Fab' and F(ab')2. It may also be a genetically engineered or synthetically produced molecule.

The novel *B. burgdorferi* polypeptides disclosed herein are immunologically reactive with antisera generated by infection of a mammalian host with *B. burgdorferi*. Accordingly, they are useful in methods and compositions to diagnose and protect against Lyme disease, and in therapeutic compositions to stimulate immunological clearance of *B. burgdorferi* during ongoing infection. In addition, because at least some, if not all of the novel *B. burgdorferi* polypeptides disclosed herein are immunogenic surface proteins of *B. burgdorferi*, they are particularly useful in a multicomponent vaccine against Lyme disease, because such a vaccine may be formulated to more closely resemble the immunogens presented by replication-competent *B. burgdorferi*, and because such a vaccine is more likely to confer broad-spectrum protection than a vaccine comprising only a single *B.*

burgdorferi polypeptide. Multicomponent vaccines according to this invention may also contain polypeptides which characterize other vaccines useful for immunization against diseases other than Lyme disease such as, for example, diphtheria, polio, hepatitis, and measles. Such multicomponent vaccines are typically incorporated into a single composition.

The preferred compositions and methods of this invention comprise novel *B. burgdorferi* polypeptides having enhanced immunogenicity. Such polypeptides may result when the native forms of the polypeptides or fragments thereof are modified or subjected to treatments to enhance their immunogenic character in the intended recipient.

Numerous techniques are available and well known to those of skill in the art which may be used, without undue experimentation, to substantially increase the immunogenicity of the novel *B. burgdorferi* polypeptides herein disclosed. For example, the polypeptides may be modified by coupling to dinitrophenol groups or arsanilic acid, or by denaturation with heat and/or SDS. Particularly if the polypeptides are small polypeptides synthesized chemically, it may be desirable to couple them to an immunogenic carrier. The coupling of course, must not interfere with the ability of either the polypeptide or the carrier to function appropriately. For a review of some general considerations in coupling strategies, see Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, ed. E. Harlow and D. Lane (1988). Useful immunogenic carriers are well known in the art. Examples of such carriers are keyhole limpet hemocyanin (KLH); albumins such as bovine serum albumin (BSA) and ovalbumin, PPD (purified protein derivative of tuberculin); red blood cells; tetanus toxoid, cholera toxoid; agarose beads; activated carbon, or bentonite.

Modification of the amino acid sequence of the novel *B. burgdorferi* polypeptides disclosed herein in order to alter the lipidation state is also a method which may be used to increase their immunogenicity and biochemical properties.

For example, the polypeptides or fragments thereof may be expressed with or without the signal sequences that direct addition of lipid moieties.

As will be apparent from the disclosure to follow, the polypeptides may also be prepared with the objective of increasing stability or rendering the molecules more amenable to purification and preparation. One such technique is to express the polypeptides as fusion proteins comprising other *B. burgdorferi* or non-*B. burgdorferi* sequences.

In accordance with this invention, derivatives of the novel *B. burgdorferi* polypeptides may be prepared by a variety of methods, including by *in vitro* manipulation of the DNA encoding the native polypeptides and subsequent expression of the modified DNA, by chemical synthesis of derivatized DNA sequences, or by chemical or biological manipulation of expressed amino acid sequences.

For example, derivatives may be produced by substitution of one or more amino acids with a different natural amino acid, an amino acid derivative or non-native amino acid, conservative substitution being preferred, e.g., 3-methylhistidine may be substituted for histidine, 4-hydroxyproline may be substituted for proline, 5-hydroxylysine may be substituted for lysine, and the like.

Causing amino acid substitutions which are less conservative may also result in desired derivatives, e.g., by causing changes in charge, conformation and other biological properties. Such substitutions would include for example, substitution of a hydrophilic residue for a hydrophobic residue, substitution of a cysteine or proline for another residue, substitution of a residue having a small side chain for a residue having a bulky side chain or substitution of a residue having a net positive charge for a residue having a net negative charge. When the result of a given substitution cannot be predicted with certainty, the derivatives may be readily assayed according to the methods disclosed herein to determine the presence or absence of the desired characteristics.

In a preferred embodiment of this invention, the novel *B. burgdorferi* polypeptides disclosed herein are prepared as part of a larger fusion protein. For example, a novel *B. burgdorferi* polypeptide of this invention may be fused at its N-terminus or C-terminus to a different immunogenic *B. burgdorferi* polypeptide, to a non-*B. burgdorferi* polypeptide or to combinations thereof, to produce fusion proteins comprising the novel *B. burgdorferi* polypeptide.

In a preferred embodiment of this invention, fusion proteins comprising novel *B. burgdorferi* polypeptides are constructed comprising B cell and/or T cell epitopes from multiple serotypic variants of *B. burgdorferi*, each variant differing from another with respect to the locations or sequences of the epitopes within the polypeptide. In a more preferred embodiment, fusion proteins are constructed which comprise one or more of the novel *B. burgdorferi* polypeptides fused to other immunogenic *B. burgdorferi* polypeptides. Such fusion proteins are particularly effective in the prevention, treatment and diagnosis of Lyme disease as caused by a wide spectrum of *B. burgdorferi* isolates.

In another preferred embodiment of this invention, the novel *B. burgdorferi* polypeptides are fused to moieties, such as immunoglobulin domains, which may increase the stability and prolong the *in vivo* plasma half-life of the polypeptide. Such fusions may be prepared without undue experimentation according to methods well known to those of skill in the art, for example, in accordance with the teachings of United States patent 4,946,778, or United States patent 5,116,964. The exact site of the fusion is not critical as long as the polypeptide retains the desired biological activity. Such determinations may be made according to the teachings herein or by other methods known to those of skill in the art.

It is preferred that the fusion proteins comprising the novel *B. burgdorferi* polypeptides be produced at the DNA level, e.g., by constructing a nucleic acid molecule encoding the fusion, transforming host cells with the

molecule, inducing the cells to express the fusion protein, and recovering the fusion protein from the cell culture. Alternatively, the fusion proteins may be produced after gene expression according to known methods.

The novel *B. burgdorferi* polypeptides may also be part of larger multimeric molecules which may be produced recombinantly or may be synthesized chemically. Such multimers may also include the polypeptides fused or coupled to moieties other than amino acids, including lipids and carbohydrates.

Preferably, the multimeric proteins will consist of multiple T or B cell epitopes or combinations thereof repeated within the same molecule, either randomly, or with spacers (amino acid or otherwise) between them.

In the most preferred embodiment of this invention, the novel *B. burgdorferi* polypeptides of this invention which are also immunogenic *B. burgdorferi* polypeptides are incorporated into a multicomponent vaccine which also comprises other immunogenic *B. burgdorferi* polypeptides. Such a multicomponent vaccine, by virtue of its ability to elicit antibodies to a variety of immunogenic *B. burgdorferi* polypeptides, will be effective to protect against Lyme disease as caused by a broad spectrum of different *B. burgdorferi* isolates, even those that may not express one or more of the Osp proteins.

The multicomponent vaccine may contain the novel *B. burgdorferi* polypeptides as part of a multimeric molecule in which the various components are covalently associated. Alternatively, it may contain multiple individual components. For example, a multicomponent vaccine may be prepared comprising two or more of the novel *B. burgdorferi* polypeptides, or comprising one novel *B. burgdorferi* polypeptide and one previously identified *B. burgdorferi* polypeptide, wherein each polypeptide is expressed and purified from independent cell cultures and the polypeptides are combined prior to or during formulation.

Alternatively, a multicomponent vaccine may be prepared from heterodimers or tetramers wherein the polypeptides have been fused to

immunoglobulin chains or portions thereof. Such a vaccine could comprise, for example, a P35 polypeptide fused to an immunoglobulin heavy chain and an OspA polypeptide fused to an immunoglobulin light chain, and could be produced by transforming a host cell with DNA encoding the heavy chain fusion and DNA encoding the light chain fusion. One of skill in the art will understand that the host cell selected should be capable of assembling the two chains appropriately. Alternatively, the heavy and light chain fusions could be produced from separate cell lines and allowed to associate after purification.

The desirability of including a particular component and the relative proportions of each component may be determined by using the assay systems disclosed herein, or by using other systems known to those in the art. Most preferably, the multicomponent vaccine will comprise numerous T cell and B cell epitopes of immunogenic *B. burgdorferi* polypeptides, including the novel *B. burgdorferi* polypeptides of this invention.

This invention also contemplates that the novel *B. burgdorferi* polypeptides of this invention, either alone or with other immunogenic *B. burgdorferi* polypeptides, may be administered to an animal via a liposome delivery system in order to enhance their stability and/or immunogenicity. Delivery of the novel *B. burgdorferi* polypeptides via liposomes may be particularly advantageous because the liposome may be internalized by phagocytic cells in the treated animal. Such cells, upon ingesting the liposome, would digest the liposomal membrane and subsequently present the polypeptides to the immune system in conjunction with other molecules required to elicit a strong immune response.

The liposome system may be any variety of unilamellar vesicles, multilamellar vesicles, or stable plurilamellar vesicles, and may be prepared and administered according to methods well known to those of skill in the art, for example in accordance with the teachings of United States patents 5,169,637, 4,762,915, 5,000,958 or 5,185,154. In addition, it may be desirable to express the

novel *B. burgdorferi* polypeptides of this invention, as well as other selected *B. burgdorferi* polypeptides, as lipoproteins, in order to enhance their binding to liposomes.

Any of the novel *B. burgdorferi* polypeptides of this invention may be used in the form of a pharmaceutically acceptable salt. Suitable acids and bases which are capable of forming salts with the polypeptides of the present invention are well known to those of skill in the art, and include inorganic and organic acids and bases.

According to this invention, we describe a method which comprises the steps of treating an animal with a therapeutically effective amount of a novel *B. burgdorferi* polypeptide, or a fusion protein or a multimeric protein comprising a novel *B. burgdorferi* polypeptide, in a manner sufficient to prevent or lessen the severity, for some period of time, of *B. burgdorferi* infection. The polypeptides that are preferred for use in such methods are those that contain protective epitopes. Such protective epitopes may be B cell epitopes, T cell epitopes, or combinations thereof.

According to another embodiment of this invention, we describe a method which comprises the steps of treating an animal with a multicomponent vaccine comprising a therapeutically effective amount of a novel *B. burgdorferi* polypeptide, or a fusion protein or multimeric protein comprising such polypeptide in a manner sufficient to prevent or lessen the severity, for some period of time, of *B. burgdorferi* infection. Again, the polypeptides, fusion proteins and multimeric proteins that are preferred for use in such methods are those that contain protective epitopes, which may be B cell epitopes, T cell epitopes, or combinations thereof.

The most preferred polypeptides, fusion proteins and multimeric proteins for use in these compositions and methods are those containing both strong T cell and B cell epitopes. Without being bound by theory, we believe that this is the best way to stimulate high titer antibodies that are effective to neutralize

B. burgdorferi infection. Such preferred polypeptides will be internalized by B cells expressing surface immunoglobulin that recognizes the B cell epitope(s). The B cells will then process the antigen and present it to T cells. The T cells will recognize the T cell epitope(s) and respond by proliferating and producing lymphokines which in turn cause B cells to differentiate into antibody producing plasma cells. Thus, in this system, a closed autocatalytic circuit exists which will result in the amplification of both B and T cell responses, leading ultimately to production of a strong immune response which includes high titer antibodies against the novel *B. burgdorferi* polypeptide.

One of skill in the art will also understand that it may be advantageous to administer the novel *B. burgdorferi* polypeptides of this invention in a form that will favor the production of T-helper cells type 2 (T_H2), which help B cells to generate antibody responses. Aside from administering epitopes which are strong B cell epitopes, the induction of T_H2 cells may also be favored by the mode of administration of the polypeptide for example by administering in certain doses or with particular adjuvants and immunomodulators, for example with interleukin-4.

To prepare the preferred polypeptides of this invention, in one embodiment, overlapping fragments of the novel *B. burgdorferi* polypeptides of this invention are constructed. The polypeptides that contain B cell epitopes may be identified in a variety of ways for example by their ability to (1) remove protective antibodies from polyclonal antiserum directed against the polypeptide or (2) elicit an immune response which is effective to prevent or lessen the severity of *B. burgdorferi* infection.

Alternatively, the polypeptides may be used to produce monoclonal antibodies which are screened for their ability to confer protection against *B. burgdorferi* infection when used to immunize naive animals. Once a given monoclonal antibody is found to confer protection, the particular epitope that is recognized by that antibody may then be identified

As recognition of T cell epitopes is MHC restricted, the polypeptides that contain T cell epitopes may be identified *in vitro* by testing them for their ability to stimulate proliferation and/or cytokine production by T cell clones generated from humans of various HLA types, from the lymph nodes, spleens, or peripheral blood lymphocytes of C3H/He mice, or from domestic animals. Compositions comprising multiple T cell epitopes recognized by individuals with different Class II antigens are useful for prevention and treatment of Lyme disease in a broad spectrum of patients.

In a preferred embodiment of the present invention, a novel *B. burgdorferi* polypeptide containing a B cell epitope is fused to one or more other immunogenic *B. burgdorferi* polypeptides containing strong T cell epitopes. The fusion protein that carries both strong T cell and B cell epitopes is able to participate in elicitation of a high titer antibody response effective to neutralize infection with *B. burgdorferi*.

Strong T cell epitopes may also be provided by non-*B. burgdorferi* molecules. For example, strong T cell epitopes have been observed in hepatitis B virus core antigen (HBcAg). Furthermore, it has been shown that linkage of one of these segments to segments of the surface antigen of Hepatitis B virus, which are poorly recognized by T cells, results in a major amplification of the anti-HBV surface antigen response, [D.R. Milich et al., "Antibody Production To The Nucleocapsid And Envelope Of The Hepatitis B Virus Primed By A Single Synthetic T Cell Site", Nature, 329, pp. 547-49 (1987)].

Therefore, in yet another preferred embodiment, B cell epitopes of the novel *B. burgdorferi* polypeptides are fused to segments of HBcAG or to other antigens which contain strong T cell epitopes to produce a fusion protein that can elicit a high titer antibody response against *B. burgdorferi*. In addition, it may be particularly advantageous to link a novel *B. burgdorferi* polypeptide of this

invention to a strong immunogen that is also widely recognized, for example tetanus toxoid.

It will be readily appreciated by one of ordinary skill in the art that the novel *B. burgdorferi* polypeptides of this invention, as well as fusion proteins and multimeric proteins containing them, may be prepared by recombinant means, chemical means, or combinations thereof.

For example, the polypeptides may be generated by recombinant means using the DNA sequences of *B. burgdorferi* strain N40 as set forth in the sequence listings contained herein. DNA encoding serotypic variants of the polypeptides may likewise be cloned, e.g., using PCR and oligonucleotide primers derived from the sequences herein disclosed.

In this regard, it may be particularly desirable to isolate the genes encoding novel *B. burgdorferi* polypeptides from strain 25015 and other strains of *B. burgdorferi* that are known to differ antigenically from strain N40, in order to obtain a broad spectrum of different epitopes which would be useful in the methods and compositions of this invention. For example, the OspA gene of *B. burgdorferi* strain 25015 is known to differ from the OspA gene of *B. burgdorferi* strain N40 to the extent that anti-OspA antibodies, which protect against subsequent infection with strain N40, appear ineffective to protect against infection with strain 25015.

Oligonucleotide primers and other nucleic acid probes derived from the genes encoding the novel *B. burgdorferi* polypeptides may also be used to isolate and clone other related surface proteins from *B. burgdorferi* and related spirochetes which may contain regions of DNA sequence homologous to the DNA sequences of this invention. In addition, the DNA sequences of this invention may also be used in PCR reactions to detect the presence of *B. burgdorferi* in a suspected infected sample.

If the novel *B. burgdorferi* polypeptides of this invention are produced recombinantly, they may be expressed in unicellular hosts. As is well

known to one of skill in the art, in order to obtain high expression levels of foreign DNA sequences in a host, the sequences are generally operatively linked to transcriptional and translational expression control sequences that are functional in the chosen host. Preferably, the expression control sequences, and the gene of interest, will be contained in an expression vector that further comprises a selection marker.

The DNA sequences encoding the polypeptides of this invention may or may not encode a signal sequence. If the expression host is eukaryotic, it generally is preferred that a signal sequence be encoded so that the mature protein is secreted from the eukaryotic host.

An amino terminal methionine may or may not be present on the expressed polypeptides of this invention. If the terminal methionine is not cleaved by the expression host, it may, if desired, be chemically removed by standard techniques.

A wide variety of expression host/vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors for eukaryotic hosts, include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus, adeno-associated virus, cytomegalovirus and retroviruses including lentiviruses. Useful expression vectors for bacterial hosts include bacterial plasmids, such as those from *E. coli*, including pBluescript, pGEX-2T, pUC vectors, col E1, pCR1, pBR322, pMB9 and their derivatives, pET-15, wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g. λGT10 and λGT11, and other phages. Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof. Useful vectors for insect cells include pVL 941.

In addition, any of a wide variety of expression control sequences -- sequences that control the expression of a DNA sequence when operatively linked to it -- may be used in these vectors to express the DNA sequences of this

invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors Examples of useful expression control sequences include, for example, the early and late promoters of SV40 or adenovirus, the lac system, the trp system, the TAC or TRC system, the T3 and T7 promoters, the major operator and promoter regions of phage lambda, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating system and other constitutive and inducible promoter sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

In a preferred embodiment, DNA sequences encoding the novel *B. burgdorferi* polypeptides of this invention are cloned in the expression vector lambda ZAP II (Stratagene, La Jolla, CA), in which expression from the lac promoter may be induced by IPTG

In another preferred embodiment, DNA encoding the novel *B. burgdorferi* polypeptides of this invention is inserted in frame into an expression vector that allows high level expression of the polypeptide as a glutathione S-transferase fusion protein. Such a fusion protein thus contains amino acids encoded by the vector sequences as well as amino acids of the novel *B. burgdorferi* polypeptide.

A wide variety of unicellular host cells are useful in expressing the DNA sequences of this invention These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi, yeast, insect cells such as *Spodoptera frugiperda* (SF9), animal cells such as CHO and mouse cells, African green monkey cells such as COS 1, COS 7, BSC 1, BSC 40, and BMT 10, and human cells, as well as plant cells.

It should of course be understood that not all vectors and expression control sequences will function equally well to express the DNA sequences of this invention. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences and hosts without undue experimentation and without departing from the scope of this invention. For example, in selecting a vector, the host must be considered because the vector must be replicated in it. The vector's copy number, the ability to control that copy number, the ability to control integration, if any, and the expression of any other proteins encoded by the vector, such as antibiotic or other selection markers, should also be considered.

In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the promoter sequence, its controllability, and its compatibility with the DNA sequence of this invention, particularly with regard to potential secondary structures. Unicellular hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the DNA sequences of this invention, their secretion characteristics, their ability to fold the polypeptide correctly, their fermentation or culture requirements, and the ease of purification from them of the products coded for by the DNA sequences of this invention.

Within these parameters, one of skill in the art may select various vector/expression control sequence/host combinations that will express the DNA sequences of this invention on fermentation or in other large scale cultures.

The molecules comprising the novel *B. burgdorferi* polypeptides encoded by the DNA sequences of this invention may be isolated from the fermentation or cell culture and purified using any of a variety of conventional methods including: liquid chromatography such as normal or reversed phase, using HPLC, FPLC and the like, affinity chromatography (such as with inorganic ligands or monoclonal antibodies), size exclusion chromatography, immobilized metal

chelate chromatography, gel electrophoresis, and the like. One of skill in the art may select the most appropriate isolation and purification techniques without departing from the scope of this invention.

In addition, the novel *B. burgdorferi* polypeptides may be generated by any of several chemical techniques. For example, they may be prepared using the solid-phase synthetic technique originally described by R. B. Merrifield, "Solid Phase Peptide Synthesis. I. The Synthesis Of A Tetrapeptide", J. Am. Chem. Soc., 83, pp. 2149-54 (1963), or they may be prepared by synthesis in solution. A summary of peptide synthesis techniques may be found in E. Gross & H. J. Meinhofer, 4 The Peptides: Analysis, Synthesis, Biology, Modern Techniques Of Peptide And Amino Acid Analysis, John Wiley & Sons, (1981) and M. Bodanszky, Principles Of Peptide Synthesis, Springer-Verlag (1984).

Typically, these synthetic methods comprise the sequential addition of one or more amino acid residues to a growing peptide chain. Often peptide coupling agents are used to facilitate this reaction. For a recitation of peptide coupling agents suitable for the uses described herein see M. Bodansky, *supra*. Normally, either the amino or carboxyl group of the first amino acid residue is protected by a suitable, selectively removable protecting group. A different protecting group is utilized for amino acids containing a reactive side group, e.g., lysine. A variety of protecting groups known in the field of peptide synthesis and recognized by conventional abbreviations therein, may be found in T. Greene, Protective Groups In Organic Synthesis, Academic Press (1981).

According to another embodiment of this invention, antibodies directed against the novel *B. burgdorferi* polypeptides are generated. Such antibodies are immunoglobulin molecules or portions thereof that are immunologically reactive with a novel *B. burgdorferi* polypeptide of the present invention. It should be understood that the antibodies of this invention include

antibodies immunologically reactive with fusion proteins and multimeric proteins comprising a novel *B. burgdorferi* polypeptide

Antibodies directed against a novel *B. burgdorferi* polypeptide may be generated by a variety of means including infection of a mammalian host with *B. burgdorferi*, or by immunization of a mammalian host with a novel *B. burgdorferi* polypeptide of the present invention. Such antibodies may be polyclonal or monoclonal, it is preferred that they are monoclonal. Methods to produce polyclonal and monoclonal antibodies are well known to those of skill in the art. For a review of such methods, see *Antibodies, A Laboratory Manual, supra*, and D.E. Yelton, et al., Ann. Rev. of Biochem., 50, pp. 657-80 (1981). Determination of immunoreactivity with a novel *B. burgdorferi* polypeptide of this invention may be made by any of several methods well known in the art, including by immunoblot assay and ELISA.

An antibody of this invention may also be a hybrid molecule formed from immunoglobulin sequences from different species (e.g., mouse and human) or from portions of immunoglobulin light and heavy chain sequences from the same species. It may be a molecule that has multiple binding specificities, such as a bifunctional antibody prepared by any one of a number of techniques known to those of skill in the art including the production of hybrid hybridomas, disulfide exchange, chemical cross-linking, addition of peptide linkers between two monoclonal antibodies, the introduction of two sets of immunoglobulin heavy and light chains into a particular cell line, and so forth.

The antibodies of this invention may also be human monoclonal antibodies produced by any of the several methods known in the art. For example, human monoclonal antibodies may produced by immortalized human cells, by SCID-hu mice or other non-human animals capable of producing "human" antibodies, by the expression of cloned human immunoglobulin genes, by phage-display, or by any other method known in the art.

In addition, it may be advantageous to couple the antibodies of this invention to toxins such as diphthena, pseudomonas exotoxin, ricin A chain, gelonin, etc., or antibiotics such as penicillins, tetracyclines and chloramphenicol.

In sum, one of skill in the art, provided with the teachings of this invention, has available a variety of methods which may be used to alter the biological properties of the antibodies of this invention including methods which would increase or decrease the stability or half-life, immunogenicity, toxicity, affinity or yield of a given antibody molecule or to alter it in any other way that may render it more suitable for a particular application.

One of skill in the art will understand that antibodies directed against a novel *B. burgdorferi* polypeptide may have utility in therapeutic and prophylactic compositions and methods directed against Lyme disease and *B. burgdorferi* infection. For example, the level of *B. burgdorferi* in infected ticks may be decreased by allowing them to feed on the blood of animals immunized with the novel *B. burgdorferi* polypeptides of this invention.

The antibodies of this invention also have a variety of other uses. For example, they are useful as reagents to screen for expression of the *B. burgdorferi* polypeptides either in libraries constructed from *B. burgdorferi* DNA or from other samples in which the proteins may be present. Moreover, by virtue of their specific binding affinities, the antibodies of this invention are also useful to purify or remove polypeptides from a given sample, to block or bind to specific epitopes on the polypeptides and to direct various molecules, such as toxins, to the surface of *B. burgdorferi*.

To screen the novel *B. burgdorferi* polypeptides and antibodies of this invention for their ability to confer protection against Lyme disease or their ability to lessen the severity of *B. burgdorferi* infection, C3H/He mice are preferred as an animal model. Of course, while any animal that is susceptible to infection with *B. burgdorferi* may be useful, C3H/He mice are not only susceptible to *B.*

burgdorferi infection but are also afflicted with clinical symptoms of a disease that is remarkably similar to Lyme disease in humans. Thus, by administering a particular polypeptide or antibody to C3H/He mice, one of skill in the art may determine without undue experimentation whether that polypeptide or antibody would be useful in the methods and compositions claimed herein.

The administration of the novel *B. burgdorferi* polypeptide or antibody of this invention to the animal may be accomplished by any of the methods disclosed herein or by a variety of other standard procedures. For a detailed discussion of such techniques, see *Antibodies: A Laboratory Manual*, *supra*. Preferably, if a polypeptide is used, it will be administered with a pharmaceutically acceptable adjuvant, such as complete or incomplete Freund's adjuvant, RIBI (muramyl dipeptides) or ISCOM (immunostimulating complexes). Such adjuvants may protect the polypeptide from rapid dispersal by sequestering it in a local deposit, or they may contain substances that stimulate the host to secrete factors that are chemotactic for macrophages and other components of the immune system. Preferably, if a polypeptide is being administered, the immunization schedule will involve two or more administrations of the polypeptide, spread out over several weeks.

Once the novel *B. burgdorferi* polypeptides or antibodies of this invention have been determined to be effective in the screening process, they may then be used in a therapeutically effective amount in pharmaceutical compositions and methods to treat or prevent Lyme disease which may occur naturally in various animals.

The pharmaceutical compositions of this invention may be in a variety of conventional depot forms. These include, for example, solid, semi-solid and liquid dosage forms, such as tablets, pills, powders, liquid solutions or suspensions, liposomes, capsules, suppositories, injectable and infusible solutions

The preferred form depends upon the intended mode of administration and prophylactic application

Such dosage forms may include pharmaceutically acceptable carriers and adjuvants which are known to those of skill in the art. These carriers and adjuvants include, for example, RIBI, ISCOM, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances, such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water salts or electrolytes such as protamine sulfate, disodium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, and polyethylene glycol. Adjuvants for topical or gel base forms may be selected from the group consisting of sodium carboxymethylcellulose, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, polyethylene glycol, and wood wax alcohols

The vaccines and compositions of this invention may also include other components or be subject to other treatments during preparation to enhance their immunogenic character or to improve their tolerance in patients.

Compositions comprising an antibody of this invention may be administered by a variety of dosage forms and regimens similar to those used for other passive immunotherapies and well known to those of skill in the art. Generally, the novel *B. burgdorferi* polypeptides may be formulated and administered to the patient using methods and compositions similar to those employed for other pharmaceutically important polypeptides (e.g., the vaccine against hepatitis)

Any pharmaceutically acceptable dosage route, including parenteral, intravenous, intramuscular, intralesional or subcutaneous injection, may be used to administer the polypeptide or antibody composition. For example, the composition may be administered to the patient in any pharmaceutically acceptable dosage form

including those which may be administered to a patient intravenously as bolus or by continued infusion over a period of hours, days, weeks or months, intramuscularly -- including paravertebrally and periarticularly -- subcutaneously, intracutaneously, intra-articularly, intrasynovially, intrathecally, intralesionally, periostally or by oral or topical routes. Preferably, the compositions of the invention are in the form of a unit dose and will usually be administered to the patient intramuscularly.

The novel *B. burgdorferi* polypeptides or antibodies of this invention may be administered to the patient at one time or over a series of treatments. The most effective mode of administration and dosage regimen will depend upon the level of immunogenicity, the particular composition and/or adjuvant used for treatment, the severity and course of the expected infection, previous therapy, the patient's health status and response to immunization, and the judgment of the treating physician. For example, in an immunocompetent patient, the more highly immunogenic the polypeptide, the lower the dosage and necessary number of immunizations. Similarly, the dosage and necessary treatment time will be lowered if the polypeptide is administered with an adjuvant. Generally, the dosage will consist of 10 μg to 100 mg of the purified polypeptide, and preferably, the dosage will consist of 10-1000 μg. Generally, the dosage for an antibody will be 0.5 mg-3.0 g.

In a preferred embodiment of this invention, the novel *B. burgdorferi* polypeptide is administered with an adjuvant, in order to increase its immunogenicity. Useful adjuvants include RIBI and ISCOM, simple metal salts such as aluminum hydroxide, and oil based adjuvants such as complete and incomplete Freund's adjuvant. When an oil based adjuvant is used, the polypeptide usually is administered in an emulsion with the adjuvant.

In yet another preferred embodiment, *E.coli* expressing proteins comprising a novel *B. burgdorferi* polypeptide are administered orally to non-

human animals to decrease or lessen the severity of *B. burgdorferi* infection. For example, a palatable regimen of bacteria expressing a novel *B. burgdorferi* polypeptide, alone or in the form of a fusion protein or multimeric protein, may be administered with animal food to be consumed by wild mice or deer, or by domestic animals. Ingestion of such bacteria may induce an immune response comprising both humoral and cell-mediated components. See J.C. Sadoff et al., "Oral *Salmonella Typhimurium* Vaccine Expressing Circumsporozoite Protein Protects Against Malaria", Science, 240, pp. 336-38 (1988) and K.S. Kim et al., "Immunization Of Chickens With Live *Escherichia coli* Expressing *Eimeria acervulina* Merozoite Recombinant Antigen Induces Partial Protection Against Coccidiosis", Inf. Immun., 57, pp. 2434-40 (1989). In fact, oral vaccination with bacteria expressing OspA has been shown to be effective. See, M. Dunne et al., "Oral Vaccination Against Lyme Disease Using *Salmonella* Expressing OspA," Inf. and Immun., 63:1611 (1995), E. Fikrig et al., "Protection of Mice From Lyme Borreliosis By Oral Vaccination With *Escherichia coli* Expressing OspA," J. Infec. Dis., 164:1224 (1991). Moreover, the level of *B. burgdorferi* infection in ticks feeding on such animals will be lessened or eliminated, thus inhibiting transmission to the next animal.

According to yet another embodiment, the antibodies of this invention as well as the novel *B. burgdorferi* polypeptides of this invention, and the DNA sequences encoding them are useful as diagnostic agents for detecting infection with *B. burgdorferi*, because the polypeptides are capable of binding to antibody molecules produced in animals, including humans that are infected with *B. burgdorferi*, and the antibodies are capable of binding to *B. burgdorferi* or antigens thereof.

Such diagnostic agents may be included in a kit which may also comprise instructions for use and other appropriate reagents, preferably a means for detecting when the polypeptide or antibody is bound. For example, the polypeptide

or antibody may be labeled with a detection means that allows for the detection of the polypeptide when it is bound to an antibody, or for the detection of the antibody when it is bound to *B. burgdorferi* or an antigen thereof.

The detection means may be a fluorescent labeling agent such as fluorescein isocyanate (FIC), fluorescein isothiocyanate (FITC), and the like, an enzyme, such as horseradish peroxidase (HRP), glucose oxidase or the like, a radioactive element such as ^{125}I or ^{51}Cr that produces gamma ray emissions, or a radioactive element that emits positrons which produce gamma rays upon encounters with electrons present in the test solution, such as ^{11}C, ^{15}O, or ^{13}N. Binding may also be detected by other methods for example via avidin-biotin complexes.

The linking of the detection means is well known in the art. For instance, monoclonal antibody molecules produced by a hybridoma can be metabolically labeled by incorporation of radioisotope-containing amino acids in the culture medium, or polypeptides may be conjugated or coupled to a detection means through activated functional groups.

The diagnostic kits of the present invention may be used to detect the presence of a quantity of *B. burgdorferi* or anti-*B. burgdorferi* antibodies in a body fluid sample such as serum, plasma or urine. Thus, in preferred embodiments, a novel *B. burgdorferi* polypeptide or an antibody of the present invention is bound to a solid support typically by adsorption from an aqueous medium. Useful solid matrices are well known in the art, and include crosslinked dextran; agarose; polystyrene, polyvinylchloride, cross-linked polyacrylamide; nitrocellulose or nylon-based materials, tubes, plates or the wells of microtiter plates. The polypeptides or antibodies of the present invention may be used as diagnostic agents in solution form or as a substantially dry powder, e.g., in lyophilized form.

Novel *B. burgdorferi* polypeptides and antibodies directed against those polypeptides provide much more specific diagnostic reagents than whole

B. burgdorferi and thus may alleviate such pitfalls as false positive and false negative results.

In particular, one of skill in the art would understand that novel *B. burgdorferi* polypeptides of this invention that are selectively expressed in the infected host and not in cultured *B. burgdorferi*, and antibodies directed against the polypeptides, allow detection of antigens and antibodies in samples that are undetectable by diagnostic methods using lysates of cultured spirochetes as the antigen.

One skilled in the art will realize that it may also be advantageous in the preparation of detection reagents to utilize epitopes from other *B. burgdorferi* proteins, including the flagella-associated protein, and antibodies directed against such epitopes. Antibodies to P35 and P37 tend to occur early in the course of *B. burgdorferi* infection while antibodies against P21 and OspF tend to appear later. Accordingly, it may be particularly advantageous to use P35 or P37 epitopes in combination with epitopes from other *B. burgdorferi* proteins that elicit antibodies that occur in the later stages of Lyme disease. Diagnostic reagents containing multiple epitopes which are reactive with antibodies appearing at different times are useful to detect the presence of anti-*B. burgdorferi* antibodies throughout the course of infection and to diagnose Lyme disease at all stages.

The polypeptides and antibodies of the present invention, and compositions and methods comprising them, may also be useful for detection, prevention, and treatment of other infections caused by spirochetes which may contain surface proteins sharing amino acid sequence or conformational similarities with the novel *B. burgdorferi* polypeptides of the present invention. These other spirochetes include *Borrelia Hermsii* and *Borrelia Recurrentis*, *Leptospira*, and *Treponema*.

According to another embodiment of this invention, we describe a method for identifying bacterial genes encoding an antigenic proteins that are

expressed during infection of a host but that are not expressed during in vitro culture of the bacteria, the method comprising the steps of:

(a) constructing an expression library for the bacteria;

(b) screening the library with antisera from an animal infected with the bacteria,

(c) screening the library with antisera from an animal immunized with non-viable bacteria or components thereof, and

(d) identifying clones that react with the first antisera but not with the second antisera.

It will be readily apparent to one of skill in the art that an expression library for use in the methods of this invention may be constructed using any techniques known in the art.

To generate antisera for use in the methods of this invention, any animal capable of generating an immune response is useful. Antisera may be generated by any of the wide variety of techniques that are well known to those of skill in the art.

As used herein, bacteria include any pathogenic or non-pathogenic bacteria that are capable of proliferating in a host. In a preferred embodiment, the bacteria are pathogenic bacteria.

As used herein, a host is any living organism that may be infected by bacteria, including plant and animal hosts. In a preferred embodiment, the host is a mammal.

As used herein, non-viable bacteria are bacteria that are incapable of synthesizing proteins. In a preferred embodiment, the bacteria are heat-killed bacteria. However, according to the methods of this invention, the bacteria may be rendered non-viable by any method known in the art.

As used herein, components of non-viable bacteria include lysates, homogenates, or subcellular fractions thereof such as cell membrane containing fractions.

To screen the expression library for clones that react with the antisera, any of the techniques known to those of skill in the art are useful. In a preferred embodiment, binding of the antisera is detected with a secondary antibody coupled to a detection means. One of skill in the art will readily appreciate that any of the wide variety of detection means known in the art is useful. Examples of useful detection means are set forth *supra*.

In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only, and are not to be construed as limiting the scope of the invention in any manner.

Example I - Construction and screening of a *B. burgdorferi* expression library

A. Construction of an Expression Library

We began with a *B. burgdorferi* genomic DNA expression library constructed in Lambda ZAP II by Stratagene (La Jolla, CA) [T. T. Lam et al., Inf. Immun., 62, pp. 290-298 (1994)]. Briefly, we grew *B. burgdorferi* strain N40 in modified Barbour-Stoenner-Kelly (BSK) II medium at 32°C for 7 days, harvested by centrifugation at 16,000 rpm for 30 minutes, and lysed with SDS [A.G. Barbour, "Isolation and Cultivation of Lyme Disease Spirochetes", Yale J. Biol. Med., 57, pp. 521-25 (1984)]. We then isolated the genomic DNA from the spirochetes and purified it by phenol/chloroform extraction.

To construct the library, 200 µg of DNA was randomly sheared, blunt-ended with S1 nuclease and the EcoR1 sites were methylated with EcoR1 methylase. EcoR1 linkers were then ligated to the ends of the DNA molecules, the DNA was digested with EcoR1 and the fragments were purified over a sucrose

gradient. Fragments of 1 to 9 kb were isolated and ligated to EcoR1 digested Lambda ZAP II arms.

We prepared *E. coli* SURE bacteria (Stratagene) for phage infection as follows. We picked a single colony into LB media supplemented with 0.2% maltose and 10 mM magnesium sulfate and cultured overnight at 30°C with vigorous shaking. We then centrifuged the cells at 2000 rpm for 10 minutes and resuspended in 10mM magnesium sulfate. The cells were further diluted to $O.D._{600} = 0.5$ for bacteriophage infection.

B. Preparation of Anti-*B. burgdorferi* Antisera

We prepared anti-*B. burgdorferi* N40 antisera for differentially screening the expression library as follows.

1. Immune Antisera

We prepared "immune" mouse anti-*B. burgdorferi* N40 antiserum as follows. We injected 3 to 5 groups of five three-week old female C3h/HeNC, or J (C3H) mice subcutaneously with an inoculum of 10^7 heat-killed (1 hour at 60° C) *B. burgdorferi* N40 in complete Freund's adjuvant (CFA). We were unable to infect mice with the heat inactivated preparation or to culture spirochetes from the preparation placed in BSKII medium, thus confirming that all of the heat-inactivated spirochetes were killed. We boosted the mice with the same dosage of *B. burgdorferi* in incomplete Freund's adjuvant (IFA) at two weeks and four weeks. Two weeks after the last boost, we sacrificed and bled the mice and separated the anti-*B. burgdorferi* antiserum by centrifuging the blood at 2000 rpm for 15 minutes.

To remove antibodies in the serum that would recognize *E. coli* and phage proteins, we absorbed the antiserum with an *E. coli*/phage lysate (Stratagene) as follows. We diluted the lysate 1:10 in Tris-buffered saline with 0.05% Tween-20 (TBST). We then incubated 0.45 µM pore size nitrocellulose filters (Millipore, Bedford, MA) in the lysate for 30 minutes at room temperature, removed and air

dried the filters on Whatman filter paper (Whatman International Ltd , Maidstone, England), and washed 3 times (5 minutes each) with TBS We blocked the filters by immersing in 1% Bovine Serum Albumin (BSA) in TBS for 1 hour at room temperature and rinsing 3 times with TBST We then diluted the mouse antiserum 1.5 in TBST, incubated it with the filters with shaking for 10 minutes at 37°C, and removed and discarded the filters

2. Infected Antisera

We injected three C3H/HeJ mice by intradermal inoculation with 10^4 *B. burgdorferi* N40 spirochetes We documented infection by culturing spirochetes from the spleen, bladder and skin (ear punches) of the challenged mice and by histopathologic examination of the joints and heart for evidence of inflammation We collected serum from the infected mice at various times after infection.

Both immune and infected antisera contained a high titer of anti-*B. burgdorferi* antibodies directed against whole cell lysates. We detected antibodies in the sera of immunized and infected mice at a dilution of 1:15,000 and 1:10,000 by immunoblot and 1:6400 and 1:3200 by ELISA, respectively Moreover, both antisera recognized many *B. burgdorferi* antigens by immunoblot, with different intensities.

After absorption, we diluted the antiserum to a final dilution of 1:100 and used it to screen the nitrocellulose filters containing the expressed proteins from the lambda ZAP library according to manufacturer's instructions.

C Differential Screening of A Genomic *B. burgdorferi* N40 Expression Library

To screen the library, we used the picoBlue Immunoscreening Kit (Stratagene) We plated 4 x 10^4 plaque forming units of recombinant phage on a lawn of bacteria, induced protein expression with 10mM IPTG and transferred the

proteins to duplicate plaque lifts on nitrocellulose filters according to methods well known in the art.

We incubated one set of plaque lifts with pooled sera from mice immunized with heat-killed spirochetes (immune sera) and the other set with sera from mice infected for nine months (infected sera). After washing, we incubated the filters with a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Organon Teknika Corp., West Chester, PA), and used nitro blue tetrazolium (NBT) (Stratagene) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Stratagene) for color development. We selected clones that reacted with infected sera but not with immune sera for further study.

Example II - Cloning of the *p21/b2* Operon

Differential screening of a *B. burgdorferi* N40 genomic expression library, as described in Example I, revealed one hundred seventy-two clones that reacted with sera from the infected mice and one hundred sixty-nine clones that reacted with sera from immunized mice. We subjected the three phage clones that reacted differentially with the two sera to another round of screening with identical results.

We excised the pBluescript plasmid from one of those clones, clone 1, by infection of XL1-Blue *E. coli* cells and rescued with R408 helper phage according to the manufacturer's instructions. Using the recovered plasmid, we used T3 and T7 universal primers to obtain an intial sequence of the plasmid. From that initial sequence of 100-300 bp, we made new primers which used to extend the sequence 100-300 bp at a time until we obtained the entire sequence.

Alternatively, we generated a nested set of deletions in the DNA insert of clone 1 with the Erase-A-Base System (Promega, Madison, WI) (using SmaI to generate the 5' blunt end and BstXI to generate a 3' overhang). We then sequenced the subclones using the Sequenase Kit (United States Biochemical Corp.,

Cleveland, OH) and reconstructed the entire sequence using MacVector (International Biotechnology, Inc , New Haven, CT)

We determined the nucleotide sequence of the plasmid insert using the Circumvent Thermal cycle Dideoxy DNA sequencing kit (New England Biolabs) Conditions for denaturation, annealing and extension were: 94° C for 30 sec., 55° C for 20 sec, and 72° C for 20 sec , respectively

Analysis of the DNA sequence of the insert revealed that we had isolated a clone containing a complete open reading frame and a partial open reading frame having the sequence set forth in SEQ ID NO: 1. We conducted a search of GenBank (December 1994) with the Genetics Computer Group Program (University of Wisconsin Biotechnology Center Madison, WI). Our search revealed that we had isolated a novel, bicistronic *B. burgdorferi* operon. We designated the complete open reading frame *p21* and the partial open reading frame *k2*. We designated the antigens encoded by the two genes in the operon P21 and K2, respectively.

Example III - Sequence analysis of the *p21/k2* operon

As shown in SEQ ID NO 1, the *p21* gene, at the 5' end of the operon, contains a 546 nucleotide open reading frame capable of encoding a 182-amino acid protein (SEQ ID NO 2) The deduced amino acid sequence of P21 contains a typical prokaryotic signal sequence for posttranslational processing by cleavage and lipidation, suggesting that the gene product is a lipoprotein of approximately 20.7 kDa. P21 has 71% amino acid sequence identity to *B. burgdorferi* OspE (Figure 7)

The ATG start codon for the *k2* gene is located 27 nucleotides downstream of the TAG stop codon of the *p21* gene The *k2* gene in clone 1 contains a partial open reading frame of 32 nucleotides, capable of encoding the first 10 amino terminal amino acids (SEQ ID NO 3) However, based on the last

two nucleotides of the K2 sequence of SEQ ID NO 3, the eleventh amino acid must be valine. Accordingly, as used herein, a K2 polypeptide is a polypeptide that comprises the 11-amino acid sequence of SEQ ID NO: 3. The amino terminal amino acids of K2 are 64% homologous with the amino terminal sequence of OspF Therefore, we would expect that the full-length protein encoded by the *k2* gene would have similar homology to full-length OspF protein.

A consensus ribosome binding site with the sequence -GGAG- (Shine-Dalgarno sequence) is located 10 bp upstream of the *p21* ATG start codon. Further upstream of this translational initiation sequence are the promoter segments known as the "-10" region and the "-35" region, which are similar to those found in *E. coli* and other *B. burgdorferi* genes. (See Figure 8 for a comparison of these regions between various *B. burgdorferi* genes). An additional ribosome binding site with the sequence -GGAG- is located 11 bp upstream of the ATG start codon of the *k2* gene. The location of these sequence elements suggests that both the *p21* and *k2* genes are controlled by a single promoter. The homology of P21 and K2 to OspE and OspF and their location in a bicistronic operon suggests that a recombinational event has most likely occurred between these genes in recent evolutionary time.

Like OspA, OspB, OspD, Osp E and OspF, the protein encoded by the *p21* gene appears to be a surface lipoprotein. As shown in SEQ ID NO. 2, the protein begins with a basic N-terminal peptide of five amino acids, followed by an amino-terminal hydrophobic domain of about 20 amino acids that corresponds to the leader peptide found in typical prokaryotic lipoprotein precursors [M.E. Brandt et al., *supra* and C.H. Wu and M. Tokunaga, "Biogenesis of Lipoproteins in Bacteria", Current Topics in Microbiology and Immunology, 125, pp. 127-157 (1986)].

The carboxyl terminus of the hydrophobic domain contains a cleavage site presumably recognized by a *B. burgdorferi* signal peptidase. In P21, as in OspF, the potential cleavage site is located between Ser_{17} and Cys_{18}.

The consensus sequence of typical bacterial lipoprotein precursors recognized and cleaved by signal peptidase II is a Leu and a Cys separated usually by two small neutral amino acids [C.H. Wu et al., *supra*]. Indeed, the OspA and OspB genes of *B. burgdorferi* B31 contain signal sequences of -L-I-A-C- and -L-I-G-C-, respectively [S. Bergstrom et al., "Molecular Analysis of Linear Plasmid-Encoded Major Surface Proteins, OspA and OspB, of the Lyme Disease Spirochaete *Borrelia burgdorferi*", Mol. Microbiol. 3, 479-86 (1989)].

In contrast, the signal sequences of the *B. burgdorferi* N40 *p21* gene (-L-I-S-S-C-), like the OspE (-L-I-G-A-C-), OspF (-L-I-V-S-C-), OspC-PKo (-L-F-I-S-C-) and OspD-B31 (-L-S-I-S-C-) genes, contains three amino acids between the leucine and cysteine instead of two. (See R.S. Fuchs et al. and S.J. Norris et al., *supra*.) However, despite this variation in the signal sequence, OspA, OspB and OspD have been shown to be lipoproteins by the established, [^{3}H]-palmitate labelling procedure. (See M.E. Brandt et al. and S.J. Norris et al., *supra*.) The leader signal sequence of P21 suggests that this surface protein may be processed as a lipoprotein as well. The addition of a lipid moiety at the cysteine residue could serve to anchor the protein to the outer surface of the spirochetes (see H.C. Wu and M. Tokunaga, *supra*).

Finally, P21 contains a long hydrophilic domain separated by short stretches of hydrophobic segments.

A comparison of the DNA sequences indicates that *p21* and *ospE* are closely related but distinct genes within the *B. burgdorferi* genome, with identical -35 and -10 promoter sequences and ribosome binding sites. The 5' upstream regions of *p21* and *ospE* are identical upstream from the -10 sequence to the boundary of the 5' flanking DNA which has been sequenced (189 nt 5' of the

ATG)(Figure 7) Only eight nucleotide differences between *p21* and *ospE* are evident in the area between the -10 region and the ATG start codon. Upstream of the ATG, the following differences are noted in *ospE*, when compared with *p21*: -54, G; -45, C, -32, T, -30, G; -24, A, -15, C; -6, T; -3, C (where -1 is the A in the ATG codon) All of the differences are located in the region likely to contain the 5' untranslated region of *p21* mRNA

In view of this homology between P21 and OspE, one of skill in the art would understand that in formulating therapeutic and diagnostic compositions, it may be desirable to select epitopes of P21 that do not cross-react with OspE

Example IV - Analysis of *p21* Expression In Cultured *B. burgdorferi* By Northern Blotting

To determine whether *p21* is transcribed during in vitro culture of spirochetes, we assessed its expression by Northern blot analysis. We isolated total RNA from cultured *B. burgdorferi* by acid guanidium thiocyanate/phenol/chloroform extraction [cite] We electrophoresed 20 µg of isolated RNA in a 1% formaldehyde-agarose gel and blotted onto Hybond-N® membrane (Amersham). We generated biotinylated *p21* and *ospA* (control) probes with a Phototope® random-primer biotin-labeling kit (New England Biolabs) The *p21* and *ospA* probes contained the entire *p21* and *ospA* sequences, respectively. We used amplified PCR products of *p21* or *ospA* as templates for the random octamer-primed labeling reaction

We conducted hybridization and signal detection with a Photope® chemiluminescent kit (New England Biolabs) Briefly, we prehybridized the blotted membrane in SSC for 1 hour at 68° C and hybridized with biotinylated probes for *p21* or *ospA* (control) at 68° C overnight We washed the membrane at a final stringency of 0 1X standard saline citrate (SSC)/0 1% SDS at 68° C We detected

biotin-labeled probe by a series of incubations with streptavidin, biotinylated alkaline phosphatase, and lumigen-PPD.

We detected *ospA* RNA but no *p21* RNA from cultured *B. burgdorferi*

Example V - Southern Dot Blot Analysis and PCR of Cultured *B. burgdorferi* Genome

Because in vitro culture of *B. burgdorferi* is often associated with the loss of genes or plasmids [cite], we used dot blot analysis and PCR to examine the genome of the cultured *B. burgdorferi* from which RNA was obtained for Northern blot analysis described in Example IV for the presence of the *p21* gene

A Southern Dot Blot Analysis

For dot blot analysis, we spotted 2 µg of denatured λ phage (control) or cultured *B. burgdorferi* DNA onto Hybond-N® membrane. We first stained the dried membrane with ethidium bromide to confirm that an equal amount of DNA was present. We then hybridized with the *p21* and *ospA* probes described in Example IV for Northern blot analysis. Both probes hybridized strongly to *B. burgdorferi* genomic DNA but not to bacteriophage DNA, confirming the presence of the *p21* gene in the cultured *B. burgdorferi*.

B PCR Analysis

We subjected 10 ng of genomic DNA from cultured *B. burgdorferi* to PCR using primers derived from the *p21* gene. We used the 5' and 3' primers shown in SEQ ID NO: 11 and SEQ ID NO: 12, respectively. These primers are specific for *p21* and do not amplify *ospE*. We used the following conditions for PCR of cultured *B. burgdorferi* DNA: 30 cycles with denaturing, annealing and extension temperatures of 94° C for 1 min., 65°C for 1 min., 72° C for 2 min., respectively

Using these primers, we obtained a 513 bp PCR product of the *p21* coding region, further confirming that the *p21* gene is present in the genome of the cultured *B. burgdorferi* used for Northern blot studies

Example VI - Examination of *p21* Expression By *B. burgdorferi* In Ticks

To determine whether P21 is expressed by *B. burgdorferi* in *Ixodes* ticks, we examined lysates of flat and engorged ticks containing the spirochetes by indirect immunofluorescence. Using the same methods, one of skill in the art could readily determine without undue experimentation whether other novel *B. burgdorferi* polypeptides of this invention are expressed in ticks

Briefly, we allowed B. burgdorferi N40-infected ticks to feed to repletion on C3H/He mice. We lightly homogenized each unfed and engorged tick in 100 µl PBS and spotted a 10 µl aliquot onto a sylilated glass slide. We air-dried the slides and fixed them with 4% paraformaldehyde and saponin. We incubated the specimens in a 1:10 dilution of antisera from mice immunized with the P21-specific peptide prepared as in Example VII and as shown in SEQ. ID NO:_, for 1 hour. We washed the slides and incubated them in anti-mouse IgG coupled to FITC (1:500 dilution) for 1 hour and viewed the slides under a Zeiss Axioskop® fluorescent microscope. We used anti-OspA monoclonal antibody CIII 78, which recognizes *B. burgdorferi* within unfed ticks but does not readily detect spirochetes within engorged ticks as a positive control [De Silva et al., (1996)] We used anti-flagellin monoclonal antibody H9724, which recognizes *B. burgdorferi* in both flat and engorged ticks as a second positive control [Cite] We used anti BSA sera as a negative control

Consistently with previous studies, spirochetes were readily detected by flagellin-specific monoclonal antibody in both flat and engorged ticks while OspA-specific monoclonal antibody detected spirochetes in flat but not in engorged

ticks. However, no P21-specific immunofluorescence was detected in either flat or engorged ticks.

To confirm that the P21-specific antisera could react with P21, we used the antisera to probe recombinant P21, prepared as in Example 12, or recombinant OspE. As expected, P21 antisera readily recognized recombinant P21 but not OspE. These results indicate that P21 is also not expressed in infected ticks.

Example VII - Confirmation of *p21* Expression in Infected Mice By Dot Immunoblot Analysis and RNA-PCR

A Dot Immunoblot Analysis

We next confirmed that *p21* is expressed in mice infected with *B. burgdorferi* by demonstrating the presence of antibodies against P21 in sera from two infected mice.

We compared the amino acid sequences of P21 and OspE and chose a region of P21 comprising amino acids 31-40 which is unique to P21. We had Quality Control Biochemicals (Hopkinton, MA) synthesize the peptide coupled to bovine serum albumin (BSA) (A cysteine was added to the amino terminus of the peptide for the BSA coupling reaction). The amino acid sequence of the peptide is set forth in SEQ ID NO. 13.

We spotted 3 µg of BSA or the synthetic P21-derived peptide coupled to BSA onto nitrocellulose membranes. We incubated the dried membranes with either serum from mice immunized with heat-killed *B. burgdorferi* or serum from infected mice. We detected bound antibody by incubating with a second antibody conjugated to horseradish peroxidase (ECL Western blot detection system, Amersham). Finally, we stained the membranes with amido black to demonstrate that an equal quantity of protein was present in all of the test samples.

Sera from infected mice but not from mice immunized with heat-killed *B. burgdorferi* reacted with the P21 peptide. Thus, P21 is selectively expressed in vivo.

B. RNA PCR

We further demonstrated expression of *p21* in infected mice using RNA PCR to detect *p21* RNA. We used acid guanidium thiocyanate/phenol/chloroform extraction (Micro RNA Isolation Kit, Stratagene) to isolate total RNA from spleens of the mice infected with *B. burgdorferi* via tick transmission and RNA from in vitro cultured *B. burgdorferi*. We allowed five *B. burgdorferi* N40-infected ticks to feed to repletion on the mice. To remove any residual DNA, we treated 10 µg of pooled RNA with RNase-free DNase (Promega) for 3 hours at 37° C with HPRI and the Rnase inhibitor. We conducted the RNA PCR with and without reverse transcriptase to exclude the possibility that residual DNA might also be amplified. We synthesized cDNA by reverse transcription with Moloney murine leukemia virus reverse transcriptase (Stratagene) and 3' primers for either *p21* (murine tissue and cultured *B. burgdorferi*), γ-actin (murine tissue control), or *ospA* (cultured *B. burgdorferi* control). We subsequently inactivated the reverse transcriptase by heating for 5 min. at 95° C. We then added 5' primer for *p21*, γ-actin or *ospA* and carried out PCR for 45 cycles of 94° C for 1 min., 55° C for 1 min. and 72° C for 2 min.

We obtained a 513 bp product from RNA PCR of *p21* only in the presence of reverse transcriptase. To confirm the identity of the amplified product as *p21*, we denatured and electrophoresed RNA PCR products, transferred them to Hybond-N® membrane and hybridized with *p21* probes as described in Example IV for Northern blot analysis. The absence of product without reverse transcriptase confirms that DNA was not amplified. We obtained no amplification with *p21*-specific primers from RNA prepared from uninfected mice or from RNA PCR of *B. burgdorferi* cultured in vitro.

Example VIII - Sequence Analysis of the p35 and *p37* Genes

We differentially screened the lambda Zap II *B. burgdorferi* expression library exactly as described in Example I but using sera from mice immunized with heat-killed *B. burgdorferi* and mice infected for 90 days with live *B. burgdorferi*. We identified 14 phage clones that reacted with antibodies in the sera from infected mice but not with antibodies in sera from mice immunized with heat-killed spirochetes

We selected two of the clones that reacted strongly to the infected antisera, excised the plasmids and sequenced the inserts as described in Example I One insert contained an open reading frame of 927 nucleotides encoding a 309 amino acid protein. (SEQ ID NO: 5) We conducted a search of GenBank (July 1995) with the Genetics Computer Group Program (University of Wisconsin Biotechnology Center, Madison, WI) Our search revealed that we had isolated a novel, *B. burgdorferi* gene which we designated *p35*. We designated the antigen encoded by the gene P35.

The other insert contained an open reading frame of 996 nucleotides encoding a 332 amino acid protein. (SEQ ID NO: 7) A search of GenBank (July 1995)revealed that we had isolated a second novel, *B. burgdorferi* gene which we designated *p37*. We designated the antigen encoded by the gene P37

As is evident from SEQ ID NO. 7, the deduced amino acid sequence of P37 reveals a leader peptide similar to those found in typical prokaryotic lipoprotein precursors At the carboxy terminus of the hydrophobic core is a potential signal peptidase II cleavage site between Ser_{19} and Cys_{20} P35, however, has a potential cleavage site with five amino acids intervening between the Leu and the Cys, making a lipoprotein less likely. It will be necessary to look for further evidence of to confirm that P35 is a lipoprotein Finally, P37 contains a long hydrophilic domain separated by short hydrophobic segments The hydrophilicity

profiles of P35 and P37, shown in Figure 6 suggest that both are hydrophilic proteins. We identified -35 and -10 regions as well as ribosome binding sites upstream of the respective open reading frames.

Example IX - Mapping of the *p21, p35* and *p37* Genes

We mapped the *p21*, *p35* and *p37* genes by pulsed-field electrophoresis (PFGE) with total *B. burgdorferi* N40 DNA using a modification of the technique described in M S. Ferdows and A.G. Barbour, "Megabase-Sized Linear DNA in the Bacterium *Borrelia burgdorferi*, the Lyme Disease Agent", Proc Natl Acad Sci., 86, pp 5969-5973 (1989). Briefly, we treated DNA plugs containing approximately 10^5 *B. burgdorferi* N40 with sarkosyl, lysed overnight with proteinase K and then separated the chromosomal and plasmid DNA by loading onto a 0 8% agarose gel. We electrophoresed the DNA in Tris-borate-EDTA (TBE) buffer (0.025 M Tris, 0.5 mM EDTA, 0 025 M boric acid) using the Chef-DRII® system (Bio-Rad Laboratories, Richmond, Calif.) at 14°C for 18 hours at 198V, with ramped pulse times from 1 to 30 sec. For two-dimensional electrophoresis of the *B. burgdorferi* DNA, we changed the direction 90 degrees and electrophoresed again at a constant voltage of 80v for 6 hours

We transferred the pulsed-field *B. burgdorferi* DNA to nitrocellulose membrane and probed with PCR-amplified radiolabelled *p21, p35, p37* probes We used *p30*, *ospA* and *ospD* probes as controls in the Southern blot. We generated *p35* and *p37* probes labeled with [a-^{32}P]dCTP, using the Prime-It® random primer kit according to the manufacturer's protocol (Stratagene)

As expected, the *ospA* and *ospD* probes hybridized to plasmids migrating at 49 kb and 38 kb, respectively [A.G. Barbour and C.F Garon, "Linear Plasmids of the Bacterium *Borrelia burgdorferi* Have Covalently Closed Ends", Science, 237, pp. 409-411 (1987) and S.J. Norris et al., *supra*]. The *p30* probe identified the chromosome The full-length *p21* probe bound at three locations but

a *p21*-specific probe (SEQ ID NO 14) recognized a circular plasmid The P35 probe bound to a plasmid which appeared to migrate with the same mobility as a linear plasmid of around 42 kb. The P37 probe bound to a plasmid which appeared to migrate with the same mobility as a linear plasmid of around 16 kb.

Example X - Analysis of Cultured *B. burgdorferi* For *p35* or *p37* Expression

To determine whether *p35* or *p37* are transcribed in vitro, we performed the same analyses as set forth in examples IV and V The 5' and 3' primers used for PCR analysis are shown in SEQ ID NOS: 15 and 16 (for *p35*) and in SEQ ID NOS 17 and 18 (for p*37*)

The results of these analyses confirmed that *p35* and *p37* are transcribed in vitro

Example XI - Confirmation of *p35* And *p37* Expression In Infected Mice by Immunoblot Analysis and RNA-PCR

We used the same dot blot and RNA PCR methods employed in Example 6 and the primers used in Example 9 We confirmed that *p35* and *p37* are expressed in infected mice Therefore, *p35* and *p37* are selectively expressed in vivo

Example XII - Expression of P21, P35 and P37 Polypeptides

To express the novel *B.* burgdorferi genes of this invention, we utilized the pMX vector, which is capable of directing expression of cloned inserts as glutathione S-transferase fusion proteins [see J. Sears et al., "Molecular Mapping of OspA-Mediated Immunity to Lyme Borreliosis", J Immunol., 147, pp 1995-2000 (1991)] The PMX vector also contains a thrombin cleavage site immediately

following the GT protein, thus, allowing the recovery of recombinant proteins without the GT fusion partner

We first used PCR to amplify the P37 gene lacking the sequences encoding the hydrophobic leader peptides. We chose to delete that sequence to ensure that the polypeptide would be expressed as soluble fusion protein rather than as a lipoprotein, which would be anchored to the cell membrane or might aggregate elsewhere in the cell during or after biosythesis

To facilitate subcloning, we amplified the genes using primers with additional restriction enzyme digestion sites. We amplified the *p21* gene using a 5' primer with an additional BamHI site and a 3'primer with a Hind III site (SEQ ID NO 21 and 22) We amplified the *p35* gene using a 5' primer with an additional XhoI restriction enzyme digestion site and a 3' primer with a supplementary Hind III site [SEQ ID NO 23 and 24]

We amplified the *p37* gene using a 5' primer with an additional BamHI restriction enzyme digestion site and a 3' primer with a supplementary XhoI site [SEQ ID NO: 25 and 26]. We used 50 ng of plasmid DNA excised from initial phage colonies using the R408 helper phage as a template for the genes

We performed the PCR for 30 cycles with initial template denaturation at 94°C for 1 minute, annealing at 40°C for 2 minutes and extension at 72°C for 3 minutes

We digested the amplified gene products with BamHI (*p21*), XhoI and Bam HI (*p35*) or Hind III and XhoI (*p37*) and cloned onto the corresponding sites in the PMX plasmid. We then used the ligation mixture to transform *Escherichia coli* DH5α according to methods well known to those of skill in the art. We isolated colonies containing the recombinant plasmid on Luria broth supplemented with ampicillin and cultured the cells

We induced expression of the genes as glutathione S-transferase fusion proteins by growing the transformed bacteria to logarithmic phase and

adding 1 mM isopropyl-1-thi-beta-galactoside (IPTG) for 3 hours One of skill in the art could readily express the other *B. burgdorferi* polypeptides of this invention without undue experimentation following the above-described techniques.

Example XIII - Purification of Recombinant Fusion Proteins

After inducing protein expression as described in Example XI, we placed the *E. coli* in phosphate buffered saline (PBS) with 1% Triton and subjected them to sonication We purified the glutathione S-transferase-*B. burgdorferi* polypeptide fusion proteins (GT-P21, GT-P35, GT-P37 and GT-M30) from cell lysates as follows.

We separated the cell supernatant and pellet by centrifugation at 1000x for 8 mins and passed the supernatant containing the recombinant fusion proteins over a glutathione-Sepharose 4B column (Pharmacia) according to the manufacturer's instructions. We eluted the fusion protein from the column using a solution containing excess glutathione and quantified using the Bradford assay.

In addition, to purify the *B. burgdorferi* proteins without the glutathione S-transferase, we loaded the glutathione S-transferase fusion proteins over the glutathione-Sepharose 4B column, added 25 units of thrombin to cleave the recombinant *B. burgdorferi* protein from the GT and incubated overnight at room temperature. We then eluted the proteins with 50 mM Tris-$CaCl_2$-NaCl, treated the eluent with anti-thrombin beads for 1 5 to 2 hours and centrifuged at 13,000 rpm.

One of skill in the art would understand that other novel *B. burgdorferi* polyeptides of this invention may be readily purified without undue experimentation using these procedures

Example XIV - Preparation Of Antibodies Directed Against The *B. burgdorferi* Polypeptides Of This Invention

We generated antibodies directed against the novel *B. burgdorferi* polypeptides of this invention as follows We immunized C3H/He mice (Frederick Cancer Research Center, Frederick, MD) subcutaneously with 10 micrograms of either GT-P21, P21-specific peptide of SEQ ID NO 13 bound to BSA, GT-P35 or GT-P37 in complete Freund's adjuvant (CFA) and boosted with the same amount of antigen in incomplete Freund's adjuvant (IFA) at 14 and 28 days according to published protocols We immunized control mice in the same manner with either recombinant glutathione S-transferase or BSA

Fourteen days after the last boost, we collected sera from the immunized animals and used it to hybridize to Western blots of SDS-PAGE gels of recombinant GT-P21, BSA-linked P21-specific peptide, P35 or P37 polypeptides

Recombinant P35 and P37 elicited antibodies in mice that were detectable by immunoblotting at a dilution of up to 1:5000 We also detected binding by ELISA.

Example XV - Isolation of the Full-Length K2 Polypeptide

The full-length K2 polypeptide and DNA encoding it may be isolated by a variety of methods available to one of skill in the art. For example, antiserum raised against the peptide set forth in SEQ ID NO 3 may be used to screen a *B. burgdorferi* expression library for clones capable of expressing the protein Alternatively, an expression library could be constructed in which smaller fragments of *B. burgdorferi* DNA are cloned in frame into an expression vector from which they would be expressed as glutathione S-transferase fusion proteins, such as pGEX-2T, pMX, or pGEMEX. Such a library would have a high likelihood of expressing the sequence as a fusion protein, even if it is normally linked to a promoter that is not transcriptionally active in *E. coli*.

Alternatively, the DNA encoding the peptide set forth in SEQ ID NO. 3 may be used as the basis of an oligonucleotide probe to screen a small cDNA library

Example XVI - Characterization of the Immune Response To Novel *B. burgdorferi* Polypeptides

A Murine Humoral Response

To characterize the immune response to the *B. burgdorferi* polypeptides of this invention, we infected C3H/He mice by intradermal inoculation with 10^4 *B. burgdorferi* N40 or by tick-transmission using *B. burgdorferi* N40 infected *I. scapularis* ticks (Harvard School of Tropical Public Health). In the tick transmission studies, we exposed mice to 5 ticks infected with *B. burgdorferi* N40. We allowed the ticks to feed to repletion and collected them over a water bath for examination.

We collected sera from infected mice at day 7, 14, 30, 90 and day 180 after infection. WE stored the samples overnight in test tubes for clot formation and isolated the sera by centrifugation for 30 min. at 900X g. We then used the sera in ELISA with purified GT-P21, BSA-linked P21-specific peptide, GT-P35 or GT-P37 polypeptides as follows

We coated duplicate sets of 96-well microtiter plates with the various recombinant polypeptides (200 micrograms {1 μg/ml, 200 ml/well} and incubated overnight at 4° C. To prevent non-specific binding, we blocked with 100 μl/ml of 10% fetal calf serum in PBS for 1 hour. We washed the plates three times with 0.05% PBS Tween (PBST). We added triplicate samples of sera (200 microliters/ well, diluted 1:100) to the coated plates and incubated for 1 hour at room temperature/ 8 hours at 4°C. We then washed the plates 3 times with PBST and added goat anti-mouse IgM or goat anti-mouse IgG, each diluted 1:2000 and linked to alkaline phosphatase, to each well. We incubated the plates at room

temperature for 1 hour and washed 3 times with PBST. Finally, we added 200 microliters of freshly prepared p-nitrophenol phosphate (1 mg/ml in glycine buffer {pH 10.5}) to each well and monitored the color change at 405 nanometers. We stopped the reaction with 3M NaOH.

We detected high titers of antibodies to both P35 and P37 as early as 14 days after infection. The response peaked 30 days after infection, diminished by 60-90 days after infection and almost disappeared by 180 days. P21-specific antibodies appeared in sera of mice on day 28 and persisted throughout the course of infection

One of skill in the art can readily determine without undue experimentation the murine humoral response to other novel *b. burgdorferi* polypeptides of this invention using the procedures taught herein

B Human Humoral Response

We also characterized the human immune response to the P21, P35 and P37 proteins. For the P21 study, we obtained a panel of 82 patients' sera from the Yale Lyme Disease Clinic and a panel of 40 patients' sera from the Centers for Disease Control (CDC). Patients were classified as having early or late stage Lyme disease based on the clinical presentation, as documented by a physician, and serum antibodies to *B. burgdorferi*, according to CDC-defined disease criteria. Over 60% of the patients that donated samples to the CDC were culture positive for *B. burgdorferi*. Patients from the Yale clinic were not routinely assessed for infection by culture.

We used the sera in ELISA with recombinant GT-P21, BSA-linked P21-specific peptide. We found that 20 of the 82 sera (24%) from the Yale clinic had IgG antibodies to recombinant P21 and 8 of those 20 also had anti-P21 IgM antibodies. Out of the 20 sera with anti-P21 antibodies, 4 had IgM and 16 had IgG antibodies that bound to P21-specific peptide. We found that 13 of the 40 sera (33%) from the CDC had IgG and/or IgM antibodies to P21. Of those 13 sera, 11

had IgG and 4 had IgM antibodies that bound to P21-specific peptide. In general, we detected IgM responses in patients with Lyme disease of 3 months or less duration. We detected IgG antibodies in patients with a disease course of greater than 3 months and in 56% of the patients with Lyme arthritis.

For the P35 and P37 studies, we used the 40 sera from the Centers for Disease Control and sera from an additional 25 patients with well-documented Lyme disease who were seen at the Yale clinic and at the Connecticut Agricultural Research Station.

We used the sera in ELISA with recombinant GT-P35 and GT-P37 as described above, using goat anti-human IgG and IgM as the secondary antibodies.

We found that all of the sera from the CDC had IgG responses to P35 and P37. Because of the high reactivity to recombinant P35 and P37, we tested sera from an additional 25 patients with well-documented Lyme disease who were seen in our clinic and Lyme disease laboratory at Yale University Medical School and the Connecticut Agricultural Research Station. Of these, 22 sera had antibody response to P35 and 20 sera had antibody response to P37.

Example XVII - Ability of Novel *B. burgdorferi* Polypeptides To Protect Against *B. burgdorferi* Infection

To determine whether the novel *B. burgdorferi* polypeptides of this invention were able to elicit an immune response that would be effective to protect against *B. burgdorferi* infection, we actively immunized C3H/He mice subcutaneously with 10 micrograms of recombinant GT-P35 or recombinant GT-P37 polypeptides in CFA and boosted at 14 and 28 days with the same amount of antigen in IFA according to published protocols. We immunized control mice in the same manner with recombinant GT. We then attempted to infect the immunized mice with *B. burgdorferi* N40.

We grew a low passage isolate of *B. burgdorferi* N40 with demonstrated infectivity and pathogenicity in C3H/He mice, to log phase at 33° C in BSK II medium and counted with a hemocytometer under dark-field microscopy We challenged the actively immunized mice approximately 14 days after the last boost with intradermal inoculations of 10^4 spirochetes and sacrificed fourteen days after infection.

At sacrifice, we aseptically collected the blood, spleen, bladder and ear punches, cultured the tissues in BSK II medium for two weeks and examined by darkfield microscopy for spirochetes At the same time we sectioned, formalin fixed and paraffin embedded, and then examined joints and hearts for inflammation. We examined the heart and tibiotarsi blindly We characterized arthritis by edema and synovial infiltration with neutrophils and lymphocytes. We characterized carditis by the presence of aortitis, myocarditis or pericarditis.

Preliminary results generated using these methods suggest that P35 or P37 may confer protection.

Example XVIII - Protection against tick-mediated transmission

We also determined whether the novel *B. burgdorferi* polypeptides of this invention were able to elicit an immune response that would be effective to protect against tick-mediated transmission of the spirochete We obtained spirochete-free *Ixodes dammini* ticks from the Harvard School of Public Health, which maintains a laboratory colony derived from an Ipswich, MA population. We infected the ticks (at the larval stage) by allowing them to feed to repletion on outbred CD-1 mice that had been previously infected (three weeks prior to serving as hosts) by intradermal inoculation of 10^3 *B. burgdorferi* N40 spirochetes. Upon repletion, we collected engorged larvae, pooled them in groups of 100-200, and permitted them to molt to the nymphal stage at 21°C and 95% relative humidity

We determined the prevalence of infection in each pool by immunofluorescence of a representative sample (10 ticks) three weeks after molting. We used only those pools having an infection prevalence of greater than 70% for challenge experiments.

We actively immunized mice with GT-P35, GT-P37, or both, GT-P21 or GT (control) as described in Example XVII. Two weeks after the last boost, we placed 5/15 infected nymphal ticks on each mouse, allowed them to feed to repletion and then allowed them to detach naturally over water. Two weeks later we sacrificed the mice and cultured the tissues for spirochetes and examine the organs, as described above.

Immunization with GT-P21 did not protect mice from infection or disease. Each mouse in the control and treatment group developed specific antibody titer of at least 1:5000 which have been found to be sufficient to protect mice from infection and disease in cases of protective antibodies like OspA (Fikrig et al., 1992). Mice were challenged with spirochetes at the peak antibody titer period which is a week after the final boost. It is possible that P21 is not expressed in high quantity in the early stages of infection. We have shown the appearance of P21-specific antibody 28 days post infection when it may be expressed in very low quantity. It is also possible that immunization with P21 did not produce sufficient protective antibodies in mice or that P21 was not expressed in sufficient quantity on the surface of the spirochete to make them vulnerable to antibody-mediated killing.

Example XIX - Decrease in spirochete load in ticks feeding on immunized animals

Previous studies have shown that immunization of mice with recombinant OspA can eliminate the spirochetes from ticks feeding on the immunized animals [E. Fikrig et al., "Elimination of *Borrelia burgdorferi* from vector ticks feeding on OspA-immunized mice", Proc. Natl. Acad. Sci., 89, pp 5418-5421 (1992)]. Thus, to determine if spirochetes also are killed when infected

ticks fed on animals immunized with the novel *B. burgdorferi* polypeptides of this invention we conduct the following experiment.

We place five *Ixodes dammini* ticks, infected as described in Example XVIII, on each of 12 control mice immunized with GT or 12 mice immunized with GT-P21. After feeding to repletion, the ticks are allowed to naturally detached over water. Only a portion of the ticks are recovered from each group, the remainder apparently having been ingested by the mice. Ten days post-repletion, we homogenized individual ticks in 100 µl of PBS in a 1.5 ml microfuge tube and spotted 10 µl aliquots on each of three slides. We allowed the slides to air-dry, fixed in cold acetone for 10 minutes, and assayed by direct or indirect immunofluorescence.

For the direct immunofluorescence assay, we incubated the slides with FITC-conjugated rabbit anti-*B. burgdorferi* N40 antiserum at a dilution of 1:100, mounted under a coverslip and examined on a Zeiss Axioscop® Fluorescent Microscope. We quantified the spirochetes by counting the number of fluorescing cells in approximately 20 fields per slide. *B. burgdorferi* infection rates were similar within ticks that fed on immunized and control mice indicating that immunization with GT-P21 does not protect against infection.

One of skill in the art would understand that the effect of immunization with other novel *B. burgdorferi* polypeptides of this invention can be readily determined without undue experimentation using the methods taught herein.

Example XX - Passive Immunization of Mice With Anti-P35 or Anti-P37 Antiserum

To determine if antiserum from animals immunized with recombinant *B. burgdorferi* polypeptides would confer protection, we passively immunized mice with 0.2 ml of GT-P35, GT-P37 or anti-P35/P37 antisera. We

then challenged the passively immunized mice with 10^4 *B. burgdorferi* N40 at one day after the immunization

Preliminary results indicate that the frequency of *B. burgdorferi* infection and the disease course in passively immunized mice appeared to be the same as in the control mice.

In a separate study, we inoculated three groups of 5 *scid* mice with 10^3 *B. burgdorferi* N40 and then injected 0.5 ml of antiserum (diluted 1:10) from either GT-P21 immunized, GT immunized or 90 day infected mice on days 1, 4, 8 and 12 post-inoculation. We sacrificed the mice on day 15 and cultured blood, bladder, spleen and skin from the inoculation site in BSK II medium. We also examined the tibiotarsi and heart of each mouse for inflammation. The rate of infection and disease in mice passively immunized with P21 antiserum was similar to the rates in control mice. Mice passive immunized with 90 day antiserum from *B. burgdorferi* infected mice were substantially protected from infection.

Again, one of skill in the art would understand that to detect a protective effect, one could various of the experimental conditions. For example, one could obtain antiserum by immunization with a recombinant polypeptide without GT, collect antiserum at a different time point when the titer is higher, passively immunize with more antiserum, decrease the spirochete dose, or other means known in the art

Example XXI - Additional Clones of In Vivo Expressed *B. burgdorferi* Polypeptides

We have performed preliminary analyses of two additional clones produced by the screening set forth in Example 1. We designated those clones V1 and V3. We deposited plasmids pV1 and pV3, contained in V1 and V3 respectively, on May 7, 1996 at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD. Clone V3 has been sequenced (SEQ ID NO. 10). One of skill in the art could conduct similar experiments as set forth above to

confirm that the polypeptides encoded by these clones are selectively expressed in vivo.

We have also performed preliminary analyses of the remaining clones identified in the screening set forth in Example VII. Based on the ability of each clone to cross-hybridize to the others, we separated those clones into five groups. At least three genes were identified in addition to those encoding P35 and P37. The DNA and amino acid sequences of one of those genes, designated M30, is set forth in SEQ ID NOS: 8 and 9. We designated the other genes J1 and J2. Plasmids from clones corresponding to J1 have been deposited as p15 and p5 under ATCC accession numbers ____. Plasmids from clones corresponding to p2, p7 and p9 have been deposited under ATCC accession numbers ____.

Example XXII - Determination of Protective Epitopes

We construct recombinant genes which will express fragments of the novel *B. burgdorferi* polypeptides in order to determine which fragments contain protective epitopes. First, we produce overlapping 200-300 bp fragments which encompass the entire nucleotide sequence of each of the genes, either by restriction enzyme digestion, or by amplification of specific sequences of using PCR and oligonucleotide primers containing restriction endonuclease recognition sequences, as described *supra*. We then clone these fragments into an appropriate expression vector, preferably a vector from which the fragments will be expressed as fusion proteins, in order to facilitate purification and increase stability. For example, the gene fragments could be cloned into pGEMEX (Promega, Madison, WS) and expressed as T7 gene 10 fusion proteins. Such proteins would be insoluble and thus easily purified by recovery of the insoluble pellet fraction followed by solubilization in denaturants such as urea. Alternatively, the fragments could be expressed as glutathione S-transferase fusion proteins as described above. We then transform appropriate host cells and induce expression of the fragments.

One way to identify fragments that contain protective B-cell epitopes is to use the individual purified fragments to immunize C3H/HeJ mice, as described above. After challenge of the mice with *B. burgdorferi*, we determine the presence of infection by blood and spleen cultures and by histopathologic examination of the joints and heart.

Another technique to identify protective epitopes is to use the various fragments to immunize mice, allow ticks infected with *B. burgdorferi* to feed on the mice, and then determine, as set forth in Example VIII, whether the immune response elicited by the fragments is sufficient to cause a decrease in the level of *B. burgdorferi* in the ticks. Any epitopes which elicit such a response, even if they are not sufficient by themselves to confer protection against subsequent infection with *B. burgdorferi*, may be useful in a multicomponent vaccine.

Once we have localized various epitopes to particular regions of the fusion proteins, we conduct further analyses using short synthetic peptides of 5-35 amino acids. The use of synthetic peptides allows us to further define each epitope, while eliminating any variables contributed by the non-*B. burgdorferi* portion of the fusion protein.

Example XXIII - Preparation of a multicomponent vaccine

We determine which of the protective epitopes is able to elicit antibodies that will protect against subsequent infection with strains of *B. burgdorferi* other than the strain from which the Osp gene was cloned. We then design a vaccine around those epitopes. If none of the protective epitopes is able to confer protection against infection with other strains of *B. burgdorferi*, it may be particularly advantageous to isolate the corresponding novel *B. burgdorferi* polypeptides from those strains. A multicomponent vaccine may then be constructed that comprises multiple epitopes from several different *B. burgdorferi*

isolates. Such a vaccine will, thus, elicit antibodies that will confer protection against a variety of different strains.

Example XXIV - Identification of T cell epitopes

Stimulation in animals of a humoral immune response containing high titer neutralizing antibodies will be facilitated by antigens containing both T cell and B cell epitopes. To identify those polypeptides containing T cell epitopes, we infect C3H/HeJ mice with *B. burgdorferi* strain N40 in complete Freund's adjuvant, as described *supra*. Ten days after priming, we harvest the lymph nodes and generate *in vitro* T cell lines. These T cell lines are then cloned using limiting dilution and soft agar techniques. We use these T cell clones to determine which polypeptides contain T cell epitopes. The T cell clones are stimulated with the various polypeptides and syngeneic antigen presenting cells. Exposure of the T cell clones to the polypeptides that contain T cell epitopes in the presence of antigen presenting cells causes the T cells to proliferate, which we measure by ^{3}H-Thymidine incorporation. We also measure lymphokine production by the stimulated T cell clones by standard methods.

To determine T cell epitopes of the polypeptides recognized by human T cells, we isolate T cell clones from *B. burgdorferi*-infected patients of multiple HLA types. T cell epitopes are identified by stimulating the clones with the various polypeptides and measuring ^{3}H-Thymidine incorporation. The various T cell epitopes are then correlated with Class II HLA antigens such as DR, DP, and DQ. The correlation is performed by utilization of B lymphoblastoid cell lines expressing various HLA genes. When a given T cell clone is mixed with the appropriate B lymphoblastoid cell line and a novel *B. burgdorferi* polypeptide, the B cell will be able to present the polypeptide to the T cell. Proliferation is then measured by ^{3}H-Thymidine incorporation.

Alternatively, T cell epitopes may be identified by adoptive transfer of T cells from mice immunized with various of the novel *B. burgdorferi* polypeptides of this invention to naive mice, according to methods well known to those of skill in the art. [See, for example, M S. DeSouza et al., "Long-Term Study of Cell-Mediated Responses to *Borrelia burgdorferi* in the Laboratory Mouse", Infect. Immun., 61, pp. 1814-22 (1993)]

We then synthesize a multicomponent vaccine based on different T cell epitopes. Such a vaccine is useful to elicit T cell responses in a broad spectrum of patients with different HLA types.

We also identify stimulating T cell epitopes in other immunogenic *B. burgdorferi* polypeptides or in non-*B. burgdorferi* polypeptides and design multicomponent vaccines based on these epitopes in conjunction with B cell and T cell epitopes from the novel *B. burgdorferi* polypeptides of this invention.

Example XXV - Construction of fusion proteins comprising T and B cell epitopes

After identifying T cell epitopes of the novel *B. burgdorferi* polypeptides, we construct recombinant proteins comprising these epitopes as well as the B cell epitopes recognized by neutralizing antibodies. These fusion proteins, by virtue of containing both T cell and B cell epitopes, permit antigen presentation to T cells by B cells expressing surface immunoglobulin. These T cells in turn stimulate B cells that express surface immunoglobin, leading to the production of high titer neutralizing antibodies.

We also construct fusion proteins from the novel *B. burgdorferi* polypeptides by linking regions of the polypeptides determined to contain B cell epitopes to strong T cell epitopes of other antigens. We synthesize an oligonucleotide homologous to amino acids 120 to 140 of the Hepatitis B virus core antigen. This region of the core antigen has been shown to contain a strong T cell epitope [D.R. Millich et al., ...

and 3' ends of segments of DNA encoding the B cell epitopes recognized by neutralizing antibodies, as in Example XI The recombinant DNA molecules are then used to express a fusion protein comprising a B cell epitope from the novel *B. burgdorferi* polypeptide and a T cell epitope from the core antigen, thus enhancing the immunogenicity of the polypeptide.

We also construct fusion proteins comprising epitopes of the novel *B. burgdorferi* polypeptides as well as epitopes of the tetanus toxoid protein

We also construct a plasmid containing the B cell epitopes of various of the novel *B. burgdorferi* polypeptides incorporated into the flagellin protein of *Salmonella*. Bacterial flagellin are potent stimulators of cellular and humoral responses, and can be used as vectors for protective antigens [S.M.C. Newton, C Jacob, B. Stocker, "Immune Response To Cholera Toxin Epitope Inserted In *Salmonella* Flagellin", Science, 244, pp. 70-72 (1989)] We cleave the cloned H 1-d flagellin gene of *Salmonella muenchens* at a unique Eco RV site in the hypervariable region. We then insert blunt ended DNAs encoding protective B cell epitopes of the polypeptides using T4 DNA ligase The recombinant plasmids are then used to transform non-flagellate strains of *Salmonella* for use as a vaccine. Mice are immunized with live and formalin killed bacteria and assayed for antibody production In addition spleen cells are tested for proliferative cellular responses to the peptide of interest. Finally the mice immunized with this agent are challenged with *B. burgdorferi* as described supra

We also construct fusion proteins comprising B cell epitopes from one of the novel *B. burgdorferi* polypeptides and T cell epitopes from a different novel *B. burgdorferi* polypeptide or other immunogenic *B. burgdorferi* polypeptides. Additionally, we construct fusion proteins comprising T cell epitopes from novel *B. burgdorferi* polypeptides and B cell epitopes from a novel *B. burgdorferi* polypeptide and/or other immunogenic *B. burgdorferi* polypeptides Construction of these fusion proteins is accomplished by recombinant DNA

techniques well known to those of skill in the art Fusion proteins and antibodies directed against them, are used in methods and composition to detect, treat, and prevent Lyme disease as caused by infection with *B. burgdorferi*

While we have described a number of embodiments of this invention, it is apparent that our basic constructions may be altered to provide other embodiments which utilize the processes and products of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the appended claims, rather than by the specific embodiments which have been presented by way of example

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Flavell, Richard A.
Fikrig, Erol
Barthold, Stephen W.
Suk, Kyoungho

(ii) TITLE OF INVENTION: B. BURGDORFERI POLYPEPTIDES EXPRESSED IN VIVO

(iii) NUMBER OF SEQUENCES: 28

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fish & Neave
(B) STREET: 1251 Avenue of the Americas
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(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 10020

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Haley Jr., James F.
(B) REGISTRATION NUMBER: 27,794
(C) REFERENCE/DOCKET NUMBER: YU-103

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212-596-9000
(B) TELEFAX: 212-596-9090

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 752 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(B) LOCATION: 721..750

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
GGAGAGATTT TTGGGAGTTG GTTAAAATTA CATTTGCGTT TTGTTAATAT GTAACAGCTG      60

AATGTAACAA AATTATATAT TTAAATCTTT CAAAAATTGT AATTATTATG TAATATGGTA     120

TGATTAAGAT TTATGGAGAA ATTT ATG AAT AAG AAA ATG TTT ATT GTT TGT         171
                          Met Asn Lys Lys Met Phe Ile Val Cys
                            1               5

GCT GTT TTT GCA CTT ATA AGT TCT TGC AAG ATT CAT ACT TTA TCT ATG       219
Ala Val Phe Ala Leu Ile Ser Ser Cys Lys Ile His Thr Leu Ser Met
 10                  15                  20                  25

TAT GAT GAG CAA AGT AAT AAT GAG TTA AAA GTT AAG CAA AGC AAT GGC       267
Tyr Asp Glu Gln Ser Asn Asn Glu Leu Lys Val Lys Gln Ser Asn Gly
                30                  35                  40

GAG GTG AAA GTT AAA AAA ATA GAA TTC TCT GAA TTT ACT GTA AAA ATA       315
Glu Val Lys Val Lys Lys Ile Glu Phe Ser Glu Phe Thr Val Lys Ile
            45                  50                  55

AAA TAT AAA AAA GAC AAT AGC AGT AAT TGG GAA GAC TTA GGA ACT TTG       363
Lys Tyr Lys Lys Asp Asn Ser Ser Asn Trp Glu Asp Leu Gly Thr Leu
        60                  65                  70

GTT GTA AGA AAA GAA GTA GAT GGT ATT GAT ACA GGG TTA AAT GTT GGG       411
Val Val Arg Lys Glu Val Asp Gly Ile Asp Thr Gly Leu Asn Val Gly
    75                  80                  85

AAG GGA TAC TCT GCT ACA TTC TTT TCA TTA GAA GAG TCA GAA GTT AAT       459
Lys Gly Tyr Ser Ala Thr Phe Phe Ser Leu Glu Glu Ser Glu Val Asn
 90                  95                  100                 105

AAC TTT ATA AAA GCA ATG ACT AAA GGT GGA ACA TTT AAA ACT AGT TTG       507
Asn Phe Ile Lys Ala Met Thr Lys Gly Gly Thr Phe Lys Thr Ser Leu
                110                 115                 120

TAT TAT GGA TAT AAG GAA GAA CAA AGT GGT GAA AAT GGT ATT CAA AAT       555
Tyr Tyr Gly Tyr Lys Glu Glu Gln Ser Gly Glu Asn Gly Ile Gln Asn
            125                 130                 135

AAG AAG ATA ATA ACA AAA ATA GAA AAA ATT GAT GAT TTT GAA TAT ATT       603
Lys Lys Ile Ile Thr Lys Ile Glu Lys Ile Asp Asp Phe Glu Tyr Ile
        140                 145                 150

ACA TTT TTA GGA GAT AAA ATT AAG GAT TCA GGA GAT AAA GTT GTT GAA       651
Thr Phe Leu Gly Asp Lys Ile Lys Asp Ser Gly Asp Lys Val Val Glu
    155                 160                 165

TAT GCA ATA CTA CTA GAA GAT CTT AAA AAA AAT TTA AAA TAGAAGTTAG       700
Tyr Ala Ile Leu Leu Glu Asp Leu Lys Lys Asn Leu Lys
170                 175                 180

AAGTATAGGG GAGAACAATT ATG AAT CAA AAA GCA TTT ATT ATT TGC GCT        750
                      Met Asn Gln Lys Ala Phe Ile Ile Cys Ala
                        1               5
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 182 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Asn Lys Lys Met Phe Ile Val Cys Ala Val Phe Ala Leu Ile Ser
 1               5                  10                  15

Ser Cys Lys Ile His Thr Leu Ser Met Tyr Asp Glu Gln Ser Asn Asn
            20                  25                  30

Glu Leu Lys Val Lys Gln Ser Asn Gly Glu Val Lys Val Lys Lys Ile
        35                  40                  45

Glu Phe Ser Glu Phe Thr Val Lys Ile Lys Tyr Lys Lys Asp Asn Ser
    50                  55                  60

Ser Asn Trp Glu Asp Leu Gly Thr Leu Val Val Arg Lys Glu Val Asp
65                  70                  75                  80

Gly Ile Asp Thr Gly Leu Asn Val Gly Lys Gly Tyr Ser Ala Thr Phe
                85                  90                  95

Phe Ser Leu Glu Glu Ser Glu Val Asn Asn Phe Ile Lys Ala Met Thr
            100                 105                 110

Lys Gly Gly Thr Phe Lys Thr Ser Leu Tyr Tyr Gly Tyr Lys Glu Glu
        115                 120                 125

Gln Ser Gly Glu Asn Gly Ile Gln Asn Lys Lys Ile Ile Thr Lys Ile
    130                 135                 140

Glu Lys Ile Asp Asp Phe Glu Tyr Ile Thr Phe Leu Gly Asp Lys Ile
145                 150                 155                 160

Lys Asp Ser Gly Asp Lys Val Val Glu Tyr Ala Ile Leu Leu Glu Asp
                165                 170                 175

Leu Lys Lys Asn Leu Lys
            180
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1353 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 181..1107

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
GGCCGATTCA TTAATGCAGC TGGCACGACA GGTTTCCCGA CTGGAAAGCG GGCAGTGAGC      60

GCAACGCAAT TAATGTGAGT TAGCTCACTC ATTAGGCACC CCAGGCTTTA CACTTTATGC     120

TTCCGGCTCG TATGTTGTGT GGAATTGTGA GCGGATAACA ATTTCACACA GGAGACAGCT     180

ATG ACC ATG ATT ACG CCA AGC TCG AAA TTA ACC CTC ACT AAA GGG AAC       228
Met Thr Met Ile Thr Pro Ser Ser Lys Leu Thr Leu Thr Lys Gly Asn
                15                  20                  25

AAA AGC TGG AGC TCC ACC GCG GTG GCG GCC GCT CTA GAA CTA GTG GAT       276
Lys Ser Trp Ser Ser Thr Ala Val Ala Ala Ala Leu Glu Leu Val Asp
            30                  35                  40

CCC CCG GGC TGC AGG AAT TCC AAA AGC AAT TTT TTA CAA AAA AAT GTA       324
Pro Pro Gly Cys Arg Asn Ser Lys Ser Asn Phe Leu Gln Lys Asn Val
        45                  50                  55

ATT TTA GAG GAA GAA AGT TTA AAA ACT GAA TTA TTA AAA GAG CAA TCT       372
Ile Leu Glu Glu Glu Ser Leu Lys Thr Glu Leu Leu Lys Glu Gln Ser
    60                  65                  70
```

```
GAG ACT AGA AAA GAA AAA ATA CAA AAA CAA CAA GAT GAA TAT AAA GGG          420
Glu Thr Arg Lys Glu Lys Ile Gln Lys Gln Gln Asp Glu Tyr Lys Gly
 75                  80                  85                  90

ATG ACT CAA GGA AGT TTA AAT TCC CTT AGC GGT GAA AGT GGT GAA TTG          468
Met Thr Gln Gly Ser Leu Asn Ser Leu Ser Gly Glu Ser Gly Glu Leu
                 95                 100                 105

GAG GAG CCT ATT GAA AGT AAT GAA ATT GAT CTT ACT ATA GAT TCT GAT          516
Glu Glu Pro Ile Glu Ser Asn Glu Ile Asp Leu Thr Ile Asp Ser Asp
            110                 115                 120

TTA AGG CCA AAG AGT TTC TTA CAA GGC ATT GCA GGA TCA AAC TCT ATT          564
Leu Arg Pro Lys Ser Phe Leu Gln Gly Ile Ala Gly Ser Asn Ser Ile
        125                 130                 135

TCA TAC ACT GAT GAA ATA GAG GAA GAG GAT TAT GAT CGC TAT TAT TTA          612
Ser Tyr Thr Asp Glu Ile Glu Glu Glu Asp Tyr Asp Arg Tyr Tyr Leu
    140                 145                 150

GAT GAA GAT GAT GAA GAT GAT GAA GAG GAT GAA GAG GAA ATA AGA TTA          660
Asp Glu Asp Asp Glu Asp Asp Glu Glu Asp Glu Glu Glu Ile Arg Leu
155                 160                 165                 170

AGC AAT CGA TAT CAA TCT TAT CTA GAA GGT GTT AAA TAT AAT GTA GAT          708
Ser Asn Arg Tyr Gln Ser Tyr Leu Glu Gly Val Lys Tyr Asn Val Asp
                175                 180                 185

TCA GCA ATT CAA ACA ATT ACT AAG ATA TAT AAT ACT TAT ACA TTA TTT          756
Ser Ala Ile Gln Thr Ile Thr Lys Ile Tyr Asn Thr Tyr Thr Leu Phe
            190                 195                 200

TCA ACA AAG CTA ACC CAA ATG TAT TCT ACA CGC CTT GAC AAC TTT GCT          804
Ser Thr Lys Leu Thr Gln Met Tyr Ser Thr Arg Leu Asp Asn Phe Ala
        205                 210                 215

AAA GCC AAA GCT AAA GAA GAA GCT GCA AAG TTT ACA AAA GAA GAC CTT          852
Lys Ala Lys Ala Lys Glu Glu Ala Ala Lys Phe Thr Lys Glu Asp Leu
    220                 225                 230

GAA AAA AAT TTC AAG ACC TTA TTA AAT TAT ATT CAA GTA AGT GTA AAG          900
Glu Lys Asn Phe Lys Thr Leu Leu Asn Tyr Ile Gln Val Ser Val Lys
235                 240                 245                 250

ACT GCA GCA AAT TTT GTA TAC ATA AAT GAC ACA CAT GCA AAA AGG AAA          948
Thr Ala Ala Asn Phe Val Tyr Ile Asn Asp Thr His Ala Lys Arg Lys
                255                 260                 265

TTA GAG AAC ATT GAA ACA GAA ATA AAA ACT TTA ATT GCA AAG ATC AAA          996
Leu Glu Asn Ile Glu Thr Glu Ile Lys Thr Leu Ile Ala Lys Ile Lys
            270                 275                 280

GAA AAA CCT GAT TTA TAC CAA GCA TAT AAA GCA ATA GTA ACG CCA ATC         1044
Glu Lys Pro Asp Leu Tyr Gln Ala Tyr Lys Ala Ile Val Thr Pro Ile
        285                 290                 295
```

```
TTA TTA ATG AGG GAT TCT CTT AAA GAA GTG CAA AGT GCC ATT GAC AAG          1092
Leu Leu Met Arg Asp Ser Leu Lys Glu Val Gln Ser Ala Ile Asp Lys
    300                 305                 310

AAT GGC ATT TGG TAC TAATTTAAGT ATTTTATTTT TAAAACAGGC TACATAATAT          1147
Asn Gly Ile Trp Tyr
315

GTAAATATGT AGCTTGTTTA AAGTAAAATA ATTAAAGTTC TAGTTGTAAA AAAGTATTGT        1207

GGATAAGAAA ATGGATTTCG TCAATTTACA AAAGGTATAT TAACTGATTT AGATAAAAGT        1267

CAAAAATATT GTTATAAGCT ATATCAGAAT TGGTAGATTG CCGTGTTTTA AAGTAAGTAT        1327

TTAGAATAGC TTTTAATATT AAGCTT                                             1353
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 309 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Thr Met Ile Thr Pro Ser Ser Lys Leu Thr Leu Thr Lys Gly Asn
 1               5                  10                  15

Lys Ser Trp Ser Ser Thr Ala Val Ala Ala Ala Leu Glu Leu Val Asp
            20                  25                  30

Pro Pro Gly Cys Arg Asn Ser Lys Ser Asn Phe Leu Gln Lys Asn Val
        35                  40                  45

Ile Leu Glu Glu Glu Ser Leu Lys Thr Glu Leu Leu Lys Glu Gln Ser
    50                  55                  60

Glu Thr Arg Lys Glu Lys Ile Gln Lys Gln Gln Asp Glu Tyr Lys Gly
65                  70                  75                  80

Met Thr Gln Gly Ser Leu Asn Ser Leu Ser Gly Glu Ser Gly Glu Leu
                85                  90                  95

Glu Glu Pro Ile Glu Ser Asn Glu Ile Asp Leu Thr Ile Asp Ser Asp
            100                 105                 110

Leu Arg Pro Lys Ser Phe Leu Gln Gly Ile Ala Gly Ser Asn Ser Ile
        115                 120                 125

Ser Tyr Thr Asp Glu Ile Glu Glu Glu Asp Tyr Asp Arg Tyr Tyr Leu
    130                 135                 140

Asp Glu Asp Asp Glu Asp Asp Glu Glu Asp Glu Glu Glu Ile Arg Leu
145                 150                 155                 160
```

Ser Asn Arg Tyr Gln Ser Tyr Leu Glu Gly Val Lys Tyr Asn Val Asp
165 170 175

Ser Ala Ile Gln Thr Ile Thr Lys Ile Tyr Asn Thr Tyr Thr Leu Phe
180 185 190

Ser Thr Lys Leu Thr Gln Met Tyr Ser Thr Arg Leu Asp Asn Phe Ala
195 200 205

Lys Ala Lys Ala Lys Glu Glu Ala Ala Lys Phe Thr Lys Glu Asp Leu
210 215 220

Glu Lys Asn Phe Lys Thr Leu Leu Asn Tyr Ile Gln Val Ser Val Lys
225 230 235 240

Thr Ala Ala Asn Phe Val Tyr Ile Asn Asp Thr His Ala Lys Arg Lys
245 250 255

Leu Glu Asn Ile Glu Thr Glu Ile Lys Thr Leu Ile Ala Lys Ile Lys
260 265 270

Glu Lys Pro Asp Leu Tyr Gln Ala Tyr Lys Ala Ile Val Thr Pro Ile
275 280 285

Leu Leu Met Arg Asp Ser Leu Lys Glu Val Gln Ser Ala Ile Asp Lys
290 295 300

Asn Gly Ile Trp Tyr
305

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1490 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 121..1116

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACTATGTTAA GTTTTATGAT ATCTATCTTA ACATCTAGCT CATAATCTTG ATTGCTACTA 60

TATATGTGAT ATAATGATAA AATATTCTAA TAATATTCTA TTTTAGATAG AGGTAATATA 120

ATG AAT TTA ATA ATT AAA GTG ATG TTG ATA TCC AGT TTA TTT TCT AGC 168
Met Asn Leu Ile Ile Lys Val Met Leu Ile Ser Ser Leu Phe Ser Ser

```
GCT TTA GCT AAA GCT TTT GTC TAT GAT AAA GAT ATA GCT GAT AAT AAA          264
Ala Leu Ala Lys Ala Phe Val Tyr Asp Lys Asp Ile Ala Asp Asn Lys
            345                 350                 355

AGT ACA AAT TCT ACT TCT AAA CTA GAT AAT AGT TCT CTA GAT TCT ATA          312
Ser Thr Asn Ser Thr Ser Lys Leu Asp Asn Ser Ser Leu Asp Ser Ile
        360                 365                 370

AAA GAC AAC AAC AGA AGT GGT CGC ACA TCT AGA GCT TTA GAT GAT GCT          360
Lys Asp Asn Asn Arg Ser Gly Arg Thr Ser Arg Ala Leu Asp Asp Ala
    375                 380                 385

GAA GAA ATT GGG GTA AAA GAA AGT AAT CAA AAC AGA AAT GAT CAA CAA          408
Glu Glu Ile Gly Val Lys Glu Ser Asn Gln Asn Arg Asn Asp Gln Gln
390                 395                 400                 405

CAA AAT AAT GAA AGT AAA GTA AAA GAA AGT GAA AAA AAC AAT AGC TCA          456
Gln Asn Asn Glu Ser Lys Val Lys Glu Ser Glu Lys Asn Asn Ser Ser
                410                 415                 420

GGT ATA CAA GCA GAT GAT AGT GTT TTA GGC ACA GCT CAT TCC GAT GCT          504
Gly Ile Gln Ala Asp Asp Ser Val Leu Gly Thr Ala His Ser Asp Ala
            425                 430                 435

AGT GAA GTA GAA AAC AAG AAA CAT GAT ACT AGC AGA CAA CCT CAA CTA          552
Ser Glu Val Glu Asn Lys Lys His Asp Thr Ser Arg Gln Pro Gln Leu
        440                 445                 450

CTT AAT AAG GAC TCT AGT GAA GCT AGA GAA GCT AGT AAA ATT ATA CAA          600
Leu Asn Lys Asp Ser Ser Glu Ala Arg Glu Ala Ser Lys Ile Ile Gln
    455                 460                 465

AAA GCT TCT ACC TCT TTA GAA GAA GCT GAG AAA GTA AAT GTG GCT TTA          648
Lys Ala Ser Thr Ser Leu Glu Glu Ala Glu Lys Val Asn Val Ala Leu
470                 475                 480                 485

AAG GAA ACA AGA TCA AAA CTT GAT AAG ATA AAA AGA TTA GCT GAT AGC          696
Lys Glu Thr Arg Ser Lys Leu Asp Lys Ile Lys Arg Leu Ala Asp Ser
                490                 495                 500

GCT AAA TCT TAT TTA AAT AAT GCT AGA AAA AAT TCT AGA ACT AAT GGT          744
Ala Lys Ser Tyr Leu Asn Asn Ala Arg Lys Asn Ser Arg Thr Asn Gly
            505                 510                 515

TCT ATA CTA GAA ATA TTG CCC AAC CTT GAT AAA GCA ATT GAA AAG GCT          792
Ser Ile Leu Glu Ile Leu Pro Asn Leu Asp Lys Ala Ile Glu Lys Ala
        520                 525                 530

ATT AGT AGT TAT GCT TCT CTT AAT GTT TGC TAT ACT GAT GCA ATT GCT          840
Ile Ser Ser Tyr Ala Ser Leu Asn Val Cys Tyr Thr Asp Ala Ile Ala
    535                 540                 545

GCT TTA GCA AAA GCT AAG AAT GAT TTT GAG CAT GCA AAA AGA AAA GCA          888
Ala Leu Ala Lys Ala Lys Asn Asp Phe Glu His Ala Lys Arg Lys Ala
550                 555                 560                 565

AAT GAT GCT TTA GAA GAA GCT TTA AAA GAT ATA ACT CAT TTT AGG GGG          936
Asn Asp Ala Leu Glu Glu Ala Leu Lys Asp Ile Thr His Phe Arg Gly
                570                 575                 580

                                                                        1032
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```
Glu Ser Ala Lys Ser Leu Leu Glu Val Ala Lys Asn Lys Gln Lys Glu
        600                 605                 610

CTT AAT GAA AAT ATT ACT AAG ACA AAT AAA GAC TTT CAA GAG TTA AAT      1080
Leu Asn Glu Asn Ile Thr Lys Thr Asn Lys Asp Phe Gln Glu Leu Asn
    615                 620                 625

GAT ATA TAT AAA AAA TTG CAA GAT ATG GAC TCT AGA TAAGTAAAAG          1126
Asp Ile Tyr Lys Lys Leu Gln Asp Met Asp Ser Arg
630                 635                 640

TAAAATATTA AAGACCAGCC AGACAATACT TTAAGAGGTT TGGCTTCTTT GTTTATAATA   1186

CTCTTTTTCT AAACAACACT TTATTTTCTC TTAACTTTAT AGTTTGACTT AAAAAGTCAT   1246

TATTTTTAAA TTATTACATG AATTGCCTTG AATATCTTTA TTTTTATATT ATAATTATTA   1306

TTAATATAGA TATTGTTTGC TATATAAGTA CATAACAAAG TTTTATTAAA AAGGAAATAT   1366

AAATATTATG CGATTATGTT TAATAAAAAT TTTTATTATA CCTAATTTAG TATTTAGTTC   1426

TCTTTTTTGA TTTGAAAGTT GTTCTGGTTT TCTATCTAAA AAATCTATAG AACAGTTTGC   1486

ATTA                                                               1490
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 332 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Met Asn Leu Ile Ile Lys Val Met Leu Ile Ser Ser Leu Phe Ser Ser
 1               5                  10                  15

Phe Ile Ser Cys Lys Leu Tyr Glu Lys Leu Thr Asn Lys Ser Gln Gln
            20                  25                  30

Ala Leu Ala Lys Ala Phe Val Tyr Asp Lys Asp Ile Ala Asp Asn Lys
        35                  40                  45

Ser Thr Asn Ser Thr Ser Lys Leu Asp Asn Ser Ser Leu Asp Ser Ile
    50                  55                  60
```

Lys Asp Asn Asn Arg Ser Gly Arg Thr Ser Arg Ala Leu Asp Asp Ala
65 70 75 80

Glu Glu Ile Gly Val Lys Glu Ser Asn Gln Asn Arg Asn Asp Gln Gln
85 90 95

Gln Asn Asn Glu Ser Lys Val Lys Glu Ser Glu Lys Asn Asn Ser Ser
100 105 110

Gly Ile Gln Ala Asp Asp Ser Val Leu Gly Thr Ala His Ser Asp Ala
115 120 125

Ser Glu Val Glu Asn Lys Lys His Asp Thr Ser Arg Gln Pro Gln Leu
130 135 140

Leu Asn Lys Asp Ser Ser Glu Ala Arg Glu Ala Ser Lys Ile Ile Gln
145 150 155 160

Lys Ala Ser Thr Ser Leu Glu Glu Ala Glu Lys Val Asn Val Ala Leu
165 170 175

Lys Glu Thr Arg Ser Lys Leu Asp Lys Ile Lys Arg Leu Ala Asp Ser
180 185 190

Ala Lys Ser Tyr Leu Asn Asn Ala Arg Lys Asn Ser Arg Thr Asn Gly
195 200 205

Ser Ile Leu Glu Ile Leu Pro Asn Leu Asp Lys Ala Ile Glu Lys Ala
210 215 220

Ile Ser Ser Tyr Ala Ser Leu Asn Val Cys Tyr Thr Asp Ala Ile Ala
225 230 235 240

Ala Leu Ala Lys Ala Lys Asn Asp Phe Glu His Ala Lys Arg Lys Ala
245 250 255

Asn Asp Ala Leu Glu Glu Ala Leu Lys Asp Ile Thr His Phe Arg Gly
260 265 270

Tyr Asn Tyr Leu Tyr His Tyr Arg Ile Asn Asn Ala Asn Asp Ala Met
275 280 285

Glu Ser Ala Lys Ser Leu Leu Glu Val Ala Lys Asn Lys Gln Lys Glu
290 295 300

Leu Asn Glu Asn Ile Thr Lys Thr Asn Lys Asp Phe Gln Glu Leu Asn
305 310 315 320

Asp Ile Tyr Lys Lys Leu Gln Asp Met Asp Ser Arg
325 330

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 825 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..825

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
ATG TGT GCT TTT TTA CTT TTA AAT TTA GTA AAT TGT AAA TTT GAT AGT          48
Met Cys Ala Phe Leu Leu Leu Asn Leu Val Asn Cys Lys Phe Asp Ser
     335                 340                 345

CTT AAT TTA TCT ACA AAA AGC GTA GAT GAT AAA AAC AAT TCT ATA GCC          96
Leu Asn Leu Ser Thr Lys Ser Val Asp Asp Lys Asn Asn Ser Ile Ala
 350                 355                 360

AAG CTT CTT CAA CAC TTA TCA AAA AGT GAA GAC CAA GCC AAT AAA ACT         144
Lys Leu Leu Gln His Leu Ser Lys Ser Glu Asp Gln Ala Asn Lys Thr
365                 370                 375                 380

TCT ACC TCA GAA GAC CAA AAG GAA TTA GAA ATT ACG GAA AAC AAA GAA         192
Ser Thr Ser Glu Asp Gln Lys Glu Leu Glu Ile Thr Glu Asn Lys Glu
                385                 390                 395

CAG GAA CAT GAA AAA CTT TCA CAA GTA GCA CAA CAT GCT CCA AAC TCA         240
Gln Glu His Glu Lys Leu Ser Gln Val Ala Gln His Ala Pro Asn Ser
            400                 405                 410

AAA ATT GAA AAA GTA AAA TCC GAT GGA AAA CCT GTT CCT GGA GAC AAA         288
Lys Ile Glu Lys Val Lys Ser Asp Gly Lys Pro Val Pro Gly Asp Lys
        415                 420                 425

ATT CTT TCT TCA AAT AAA GAT ATT TAC AAT TCT TAT ATC CCA GAA GTA         336
Ile Leu Ser Ser Asn Lys Asp Ile Tyr Asn Ser Tyr Ile Pro Glu Val
    430                 435                 440

AAA GAG GAA ATT GTT TAT GAA ATT CTT GAA GAA GTG ATA ATT CCC GAA         384
Lys Glu Glu Ile Val Tyr Glu Ile Leu Glu Glu Val Ile Ile Pro Glu
445                 450                 455                 460

ACA AAA ATT CCT GAA ATT ACT GAA GAA GTG ATA ATG CCT ATT CCA CAA         432
Thr Lys Ile Pro Glu Ile Thr Glu Glu Val Ile Met Pro Ile Pro Gln
                465                 470                 475

ACA ATA GAT TTT TAT ATT GAA CCA AGG CCA ATA AGT AGT TTC CTT ACT         480
Thr Ile Asp Phe Tyr Ile Glu Pro Arg Pro Ile Ser Ser Phe Leu Thr
            480                 485                 490

CAA GGG ACA TCA CCA AGT ATT ACA AGT ACA ATA AAA TCA TAT AAA GAA         528
Gln Gly Thr Ser Pro Ser Ile Thr Ser Thr Ile Lys Ser Tyr Lys Glu
        495                 500                 505
```

```
CTC GCT AAA GAA AAA ATT AAT AAT GGC TTG AAT ATA GTA CAG AAA ATA          576
Leu Ala Lys Glu Lys Ile Asn Asn Gly Leu Asn Ile Val Gln Lys Ile
    510                 515                 520

ACT CAA AAT ATT GAT AAT ATT ACA GAA AAT TTA AAT TCT AAA GAA ACA          624
Thr Gln Asn Ile Asp Asn Ile Thr Glu Asn Leu Asn Ser Lys Glu Thr
525                 530                 535                 540

CCA AAG GAA ATA TCG GGA AAA GAA GTT GAA GAA AAA ATT ACA CAC CCC          672
Pro Lys Glu Ile Ser Gly Lys Glu Val Glu Glu Lys Ile Thr His Pro
                545                 550                 555

ATA TTT GAT CAC ATT ACT GGA AGC GGT AAT AAT CCC GGA CAA GAT TCT          720
Ile Phe Asp His Ile Thr Gly Ser Gly Asn Asn Pro Gly Gln Asp Ser
            560                 565                 570

ATA TCC AAT ACA TGG GGC GAA GGA CTT GAA ATT GGT GGT GAT AGC AAT          768
Ile Ser Asn Thr Trp Gly Glu Gly Leu Glu Ile Gly Gly Asp Ser Asn
        575                 580                 585

TTC TTT ACC AAT TTA GAA GAA GTA AGA AGC TCT ATA AGA ACA AAA ATC          816
Phe Phe Thr Asn Leu Glu Glu Val Arg Ser Ser Ile Arg Thr Lys Ile
    590                 595                 600

AAA GTT TCT                                                              825
Lys Val Ser
605
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 275 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Met Cys Ala Phe Leu Leu Asn Leu Val Asn Cys Lys Phe Asp Ser
 1               5                  10                  15

Leu Asn Leu Ser Thr Lys Ser Val Asp Asp Lys Asn Asn Ser Ile Ala
            20                  25                  30

Lys Leu Leu Gln His Leu Ser Lys Ser Glu Asp Gln Ala Asn Lys Thr
        35                  40                  45

Ser Thr Ser Glu Asp Gln Lys Glu Leu Glu Ile Thr Glu Asn Lys Glu
    50                  55                  60

Gln Glu His Glu Lys Leu Ser Gln Val Ala Gln His Ala Pro Asn Ser
65                  70                  75                  80

Lys Ile Glu Lys Val Lys Ser Asp Gly Lys Pro Val Pro Gly Asp Lys
                85                  90                  95
```

Ile Leu Ser Ser Asn Lys Asp Ile Tyr Asn Ser Tyr Ile Pro Glu Val
100 105 110

Lys Glu Glu Ile Val Tyr Glu Ile Leu Glu Glu Val Ile Ile Pro Glu
115 120 125

Thr Lys Ile Pro Glu Ile Thr Glu Glu Val Ile Met Pro Ile Pro Gln
130 135 140

Thr Ile Asp Phe Tyr Ile Glu Pro Arg Pro Ile Ser Ser Phe Leu Thr
145 150 155 160

Gln Gly Thr Ser Pro Ser Ile Thr Ser Thr Ile Lys Ser Tyr Lys Glu
165 170 175

Leu Ala Lys Glu Lys Ile Asn Asn Gly Leu Asn Ile Val Gln Lys Ile
180 185 190

Thr Gln Asn Ile Asp Asn Ile Thr Glu Asn Leu Asn Ser Lys Glu Thr
195 200 205

Pro Lys Glu Ile Ser Gly Lys Glu Val Glu Glu Lys Ile Thr His Pro
210 215 220

Ile Phe Asp His Ile Thr Gly Ser Gly Asn Asn Pro Gly Gln Asp Ser
225 230 235 240

Ile Ser Asn Thr Trp Gly Glu Gly Leu Glu Ile Gly Gly Asp Ser Asn
245 250 255

Phe Phe Thr Asn Leu Glu Glu Val Arg Ser Ser Ile Arg Thr Lys Ile
260 265 270

Lys Val Ser
275

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1221 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAATTCCGTG GTGAGCAGGA TGGTAAGGCG CCTGGTGATG CTAGAAATCC GATTGCGCGC 60

[illegible]

[illegible]GCTAATGCT GATGGTGCAA ATGTGAAGAG [illegible]

TGGTGCACTA AAAGATGTTC AAGCTGCTGC TGCTGATGCT GCAGAAGCGG GGAAATTGTT 300

TGGTGCTGGT GGTGGTAATG CTAATGCTGA TGATATTAAG AAGGCGGCTG AGGCTGTTAG 360

TTCGGTTAGT GGGAAGCAGA TACTAATACT TCAGTAGATA AAAATAGTAA GGAAATTGAA 420

TCTCCTAAAG ACGTTACATC ATCAAATAAA AAAACTTATG ATCCAATCTT ACAAGTAGGT 480

TCTAATCAAC ATATGTCAGA TGATCCTGGT GCAAATAATA AAGAATCCCT ACCAAATTCC 540

AGTCCAGCAA TAATACAAAA TGACTCGCAT GCTCAAAATA ATGTAAAGAT GGAAGAAAAT 600

AAATCAGCTA CTCCACAACA TGATCCAATT GAACAAAGTA ATTTTAAAAA TAGCCTTACT 660

ACAACAAGTA AAACTCCTGC TATTCCTTCA GAAAAAGAAA TTAAAGCTAA CTTAGATGAA 720

TTTGCACAAG AAGAGTATGA GCAAACATCT CTTTCAGAAA TTAAAAATGC CACGCAAATT 780

GTTAATCATG CTAATCCTGA AAACAAATTA AACAATACAC TCCTTGAGTT TGAAAAAGAT 840

TATGAAACTT TATCAAACTT GTTATTCTCT AATTTAGACA CATCTCCTTT GAATAGAAAA 900

ATAAAGACTA TTATGCCTAA ATTACAAGAA ATGCGTTCTT TTATGGAGCA AGCAACTAAT 960

TCTTGGGTAT CTGCTAAAGG CATGCTAGAT GGGGCTAAGG ATAAACTAGC AGAATCTATT 1020

TATAAAAGAC TATACAATGG CAATTCATAC CGGTTCGGTG GCAGTTTTAA CGGACGTGAT 1080

ATGCAACATG CAAAAAATTT AGCATACAGA GCTATAGACT TTGCTTCTGC ATGCATTGAA 1140

TATACACAAA AAGCTATTGA TTATCTTCAA CAGGGAAATT CTTGCAAAAA AGAAATAGAA 1200

AATATATTCA AGCTGGAATT C 1221

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGATCCTCGA GAAGATTCAT ACTTTATCTA TG 32

(2) INFORMATION FOR SEQ ID NO:12:

[illegible]

STRANDEDNESS [illegible]

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGTACAAGCT TCTATTTTAA ATTTTTTTTA AGATC 35

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Cys Asn Asn Glu Leu Lys Val Lys Gln Ser Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCTGATTTAA GGCCAAAG 18

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CATTTGGGTT AGCTTTGT 18

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CATGATACTA GCAGACAA 18

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTAGAGTCCA TATCTTGC 18

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCTGACGATC TAGGTCAAAC CACA 24

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

[illegible]

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCCTCTAATT TGGTGCCATT TG 22

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGTCGGATCC AAGATTCATA CTTTATCGAT G 31

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AGTCAAGCTT CTATTTTAAA TTTTTTTTAA GATC 34

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CCCTCGAGAT GACCATGATT ACGCCA 26

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGAAGCTTTT AGTACCAAAT GCCATT 26

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GAGGATCCAA ATTATATGAA AAGCTTAC 28

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GAGGATCCAT GAATTTAATA ATTAAAGT 28

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TTCACAAGTA GCACAACATG CTCC 24

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

[illegible]

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page _____ line _____

B. IDENTIFICATION OF DEPOSIT plasmid p-p21-k2 Further deposits are identified on an additional sheet [X]

Name of depositary institution
American Type Culture Collection

Address of depositary institution (*including postal code and country*)
12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit
7 May 1996 (07.05.96)

Accession Number

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) This information is continued on an additional sheet [ ]

In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by the requester (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

EP

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indications listed below will be submitted to the International Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

Accession number of deposit.

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 16 line 22

B. IDENTIFICATION OF DEPOSIT Plasmid p-V1 Further deposits are identified on an additional sheet [X]

Name of depositary institution
American Type Culture Collection

Address of depositary institution (including postal code and country)
12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit
7 May 1996 (07.05.96)

Accession Number

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet [ ]

In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by the requester (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

EP

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

Accession number of deposit.

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[X] This sheet was received with the international application

Authorized officer

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Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page p.16, lin.20, line

B. IDENTIFICATION OF DEPOSIT plasmid p-V3 Further deposits are identified on an additional sheet [X]

Name of depositary institution
American Type Culture Collection

Address of depositary institution *(including postal code and country)*
12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit
7 May 1996 (07.05.96)

Accession Number

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by the requester (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

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E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page p17, line 27 ; page 66, line 10

B. **IDENTIFICATION OF DEPOSIT** plasmid p 15 Further deposits are identified on an additional sheet [X]

Name of depositary institution

American Type Culture Collection

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit: 7 May 1996 (07.05.96)

Accession Number:

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by the requester (Rule 28(4) EPC).

D. **DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** *(if the indications are not for all designated States)*

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E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page p. 17, line 16 ; page 68, line 9

B. IDENTIFICATION OF DEPOSIT plasmid p 5 Further deposits are identified on an additional sheet [X]

Name of depositary institution
American Type Culture Collection

Address of depositary institution (*including postal code and country*)
12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit
7 May 1996 (07.05.96)

Accession Number

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) This information is continued on an additional sheet [ ]

In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by the requester (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

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E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page p 17, line 18 and p 68, line 10

B. IDENTIFICATION OF DEPOSIT Plasmid p 2 Further deposits are identified on an additional sheet [X]

Name of depositary institution

American Type Culture Collection

Address of depositary institution (*including postal code and country*)

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit: 7 May 1996 (07.05.96)

Accession Number:

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) This information is continued on an additional sheet [ ]

In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by the requester (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 18, line 11 ~~p. 66~~ p. 66, line 10

B. IDENTIFICATION OF DEPOSIT plasmid p 7 Further deposits are identified on an additional sheet [X]

Name of depositary institution
American Type Culture Collection

Address of depositary institution (including postal code and country)
12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit
7 May 1996 (07.05.96)

Accession Number

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet [ ]

In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by the requester (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

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E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page p. 18, line 11 and/or p. 68, line 10

B. IDENTIFICATION OF DEPOSIT plasmid p 9 Further deposits are identified on an additional sheet ☐

Name of depositary institution
American Type Culture Collection

Address of depositary institution (including postal code and country)
12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit
7 May 1996 (07.05.96)

Accession Number

C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet ☐

In respect of the designation of the EPO, samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application is refused or withdrawn or is deemed to be withdrawn, as provided in Rule 28(3) of the Implementing Regulations under the EPC only by the issue of a sample to an expert nominated by the requester (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

EP

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications (e.g., "Accession Number of Deposit")

Accession number of deposit.

For receiving Office use only

☑ This sheet was received with the international application

Authorized officer

For International Bureau use only

☐ This sheet was received by the International Bureau on

Authorized officer

We claim

1 An isolated DNA molecule comprising a DNA sequence which encodes a *B. burgdorferi* polypeptide, wherein said polypeptide is selected from the group consisting of:

(a) a P35 polypeptide encoded by SEQ ID NO: 4,

(b) a P37 polypeptide encoded by SEQ ID NO: 6,

(c) an M30 polypeptide encoded by SEQ ID NO: 8,

(d) a V3 polypeptide encoded by SEQ ID NO: 10,

(e) a J1 polypeptide encoded in whole or in part by the *B. burgdorferi* DNA sequence contained within ATCC deposit #_,

(f) a J2 polypeptide encoded in whole or in part by the *B. burgdorferi* DNA sequence contained within ATCC deposit #_,

(g) serotypic variants of any one of the polypeptides of (a)-(f);

(h) fragments comprising at least 8 amino acids taken as a block from any one of the polypeptides of (a)-(g),

(i) derivatives of any one of the polypeptides of (a)-(h), said derivatives being at least 80% identical in amino acid sequence to the corresponding polypeptide of (a)-(h);

(j) polypeptides that are immunologically reactive with antibodies generated by infection of a mammalian host with *B. burgdorferi*, which antibodies are immunologically reactive with any one of the polypeptides of (a)-(i),

(k) polypeptides that are capable of eliciting antibodies that are immunologically reactive with *B. burgdorferi* and any one of the polypeptides of (a)-(i), and

(l) polypeptides that are immunologically reactive with antibodies elicited by immunization with any one of the polypeptides of (a)-(i).

2. An isolated DNA molecule comprising a DNA sequence which encodes a *B. burgdorferi* polypeptide, wherein said polypeptide is selected from the group consisting of:

(a) a P21 polypeptide consisting of amino acids 1-182 of SEQ ID NO: 2

(b) fragments comprising at least 15 amino acids taken as a block from the P21 polypeptide of (a); and

(c) a polypeptide that is selectively expressed in vivo and that

(1) is a derivative of a P21 polyeptide of (a), said derivative being at least 80% identical in amino acid sequence to the corresponding polypeptide of (a);

(2) polypeptides that are immunologically reactive with antibodies generated by infection of a mammalian host with *B. burgdorferi*, which antibodies are immunologically reactive with a P21 polypeptide of (a);

(3) polypeptides that are capable of eliciting antibodies that are immunologically reactive with *B. burgdorferi* and the P21 polypeptide of (a); and

(4) polypeptides that are immunologically reactive with antibodies elicited by immunization with the P21 polypeptide of (a).

3 An isolated DNA molecule comprising a DNA sequence which encodes a *B. burgdorferi* polypeptide, wherein said polypeptide is selected from the group consisting of:

(a) a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 3;

(b) derivatives of the polypeptide of (a), said derivative comprising a polypeptide having a block of amino acids at least 80% identical in sequence to SEQ ID NO: 3; and

(c) a polypeptide that is selectively expressed in vivo and that

(1) is a derivative of a polyeptide of (a), said derivative being at least 80% identical in amino acid sequence to the corresponding polypeptide of (a),

(2) polypeptides that are immunologically reactive with antibodies generated by infection of a mammalian host with *B. burgdorferi*, which antibodies are immunologically reactive with a polypeptide of (a),

(3) polypeptides that are capable of eliciting antibodies that are immunologically reactive with *B. burgdorferi* and the polypeptide of (a), and

(4) polypeptides that are immunologically reactive with antibodies elicited by immunization with the polypeptide of (a).

4 The DNA molecule according to any one of claims 1 to 3, wherein the polypeptide comprises a protective epitope.

5. An isolated DNA molecule comprising a DNA sequence encoding a fusion protein comprising a *B. burgdorferi* polypeptide according to any one of claims 1 to 4.

6 An isolated DNA molecule comprising a DNA sequence encoding a multimeric protein, which multimeric protein comprises a *B. burgdorferi* polypeptide according to any one of claims 1 to 4.

7 A DNA molecule according to any one of claims 1-6, further comprising an expression control sequence operatively linked to the DNA sequence

8. A host cell transformed with a DNA molecule according to any one of claims 1 to 7

9. A polypeptide encoded by a DNA molecule according to any one of claims 1 to 6

10. A method for producing a polypeptide according to claim 9, comprising the step of culturing a host cell transformed with a DNA molecule according to claim 7

11. A fusion protein comprising a *B. burgdorferi* polypeptide according to claim 9

12. The fusion protein according to claim 11, wherein said fusion protein comprises two or more *B. burgdorferi* polypeptides according to claim 9, each derived from a different strain of *B. burgdorferi*

13 The fusion protein according to claim 11, wherein said fusion protein further comprises an immunogenic *B. burgdorferi* polypeptide different than the polypeptide according to claim 9

14. A multimeric protein comprising a polypeptide according to claim 9.

15. An antibody that binds to a polypeptide according to claim 9

16. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a component selected from the group consisting of: a polypeptide according to claim 9; a fusion protein according to any one of claims 11 to 13, and a multimeric protein according to claim 14.

17. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of an antibody according to claim 15.

18. The pharmaceutical composition according to claim 16, further comprising at least one additional immunogenic *B. burgdorferi* polypeptide.

19. The pharmaceutical composition according to claim 16, further comprising at least one additional non-*B. burgdorferi* polypeptide.

20 A method for treating or preventing *B. burgdorferi* infection or Lyme disease comprising the step of administering to a patient a therapeutically effective amount of a pharmaceutical composition according to any one of claims 16 to 19

21 A diagnostic kit comprising a component selected from the group consisting of: a polypeptide according to claim 9; a fusion protein according to any one of claims 11-13; and a multimeric protein according to claim 14, and also comprising a means for detecting binding of said component to an antibody

22 A method for detecting *B. burgdorferi* infection comprising the step of contacting a body fluid of a suspected infected mammalian host with a polypeptide according to claim 9; a fusion protein according to any one of claims 11-13; and a multimeric protein according to claim 14

23 A diagnostic kit comprising an antibody according to claim 15

24 A method for detecting *B. burgdorferi* infection comprising the step of contacting a body fluid of a mammalian host with an antibody according to claim 15

25 A method for identifying a bacterial gene encoding an antigenic protein which is expressed during infection of a host but is not expressed during in vitro culture of the bacteria, comprising the steps of:

(a) constructing an expression library from the bacterial DNA;

(b) screening the expression library with a first antiserum from an animal infected with the bacteria;

(c) screening the expression library with a second antiserum from an animal immunized with non-viable bacteria or components thereof; and

(d) identifying clones that react with the first antiserum but not with the second antiserum

26 The method according to claim 25, wherein the non-viable bacteria is obtained from in vitro culture of the bacteria.

27. The method according to claim 25, wherein the non-viable bacteria is obtained from an infected host vector

28 The method according to any one of claims 25 to 27, wherein the bacteria is a spirochete.

29. The method according to claim 28, wherein the bacteria is *B. burgdorferi*

30. The method according to claim 29, wherein the host is a tick

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Figure 1

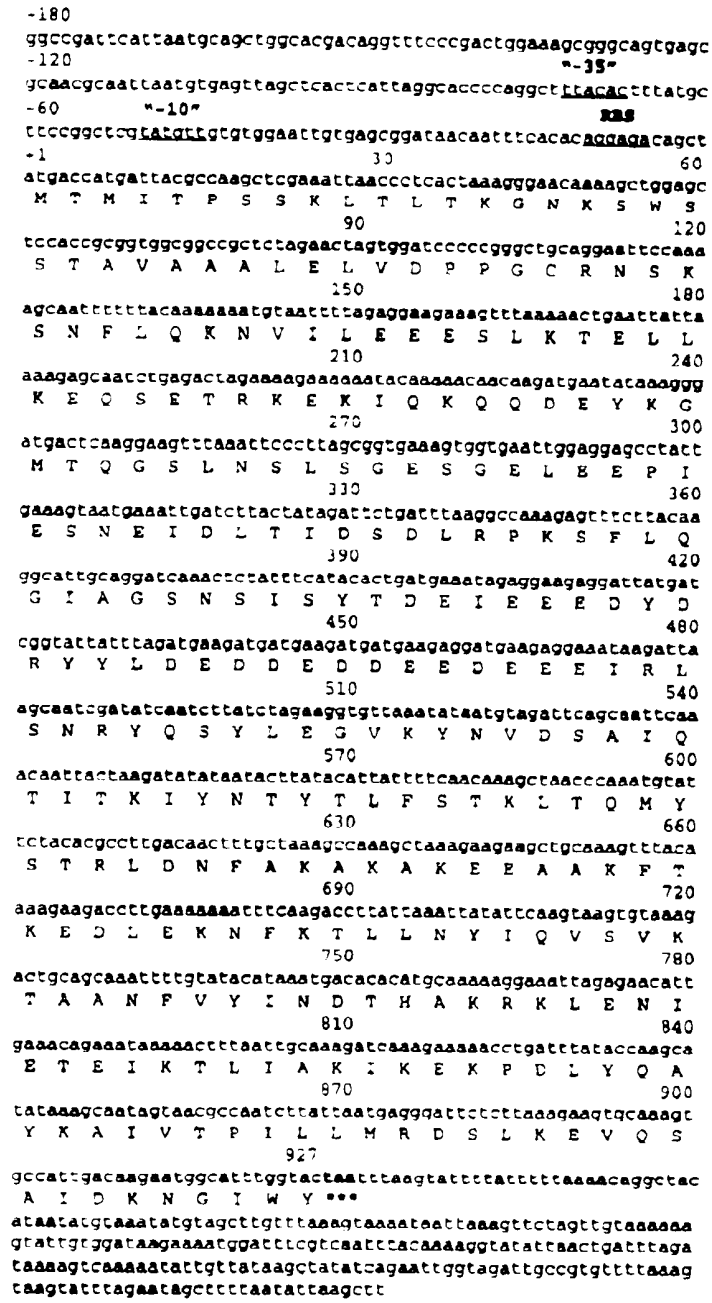
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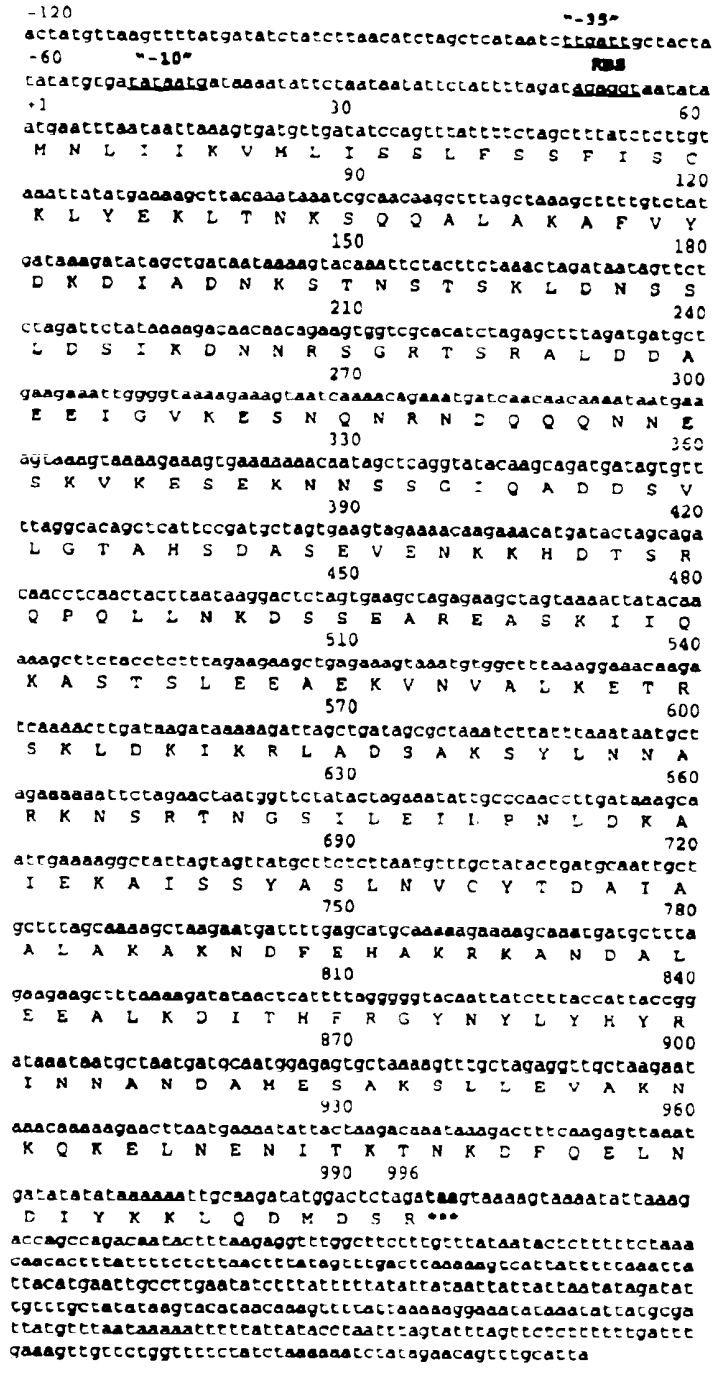
Figure 2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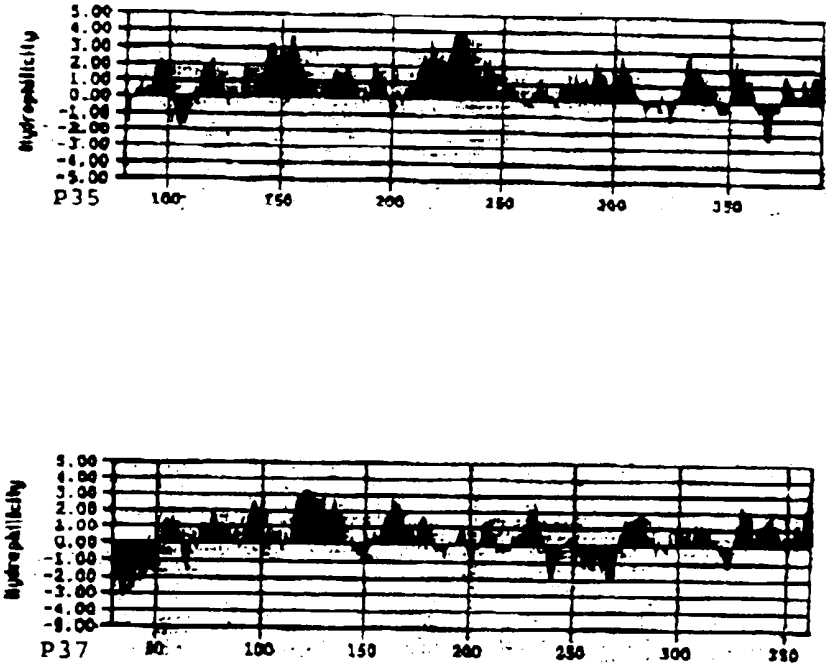

Figure 6

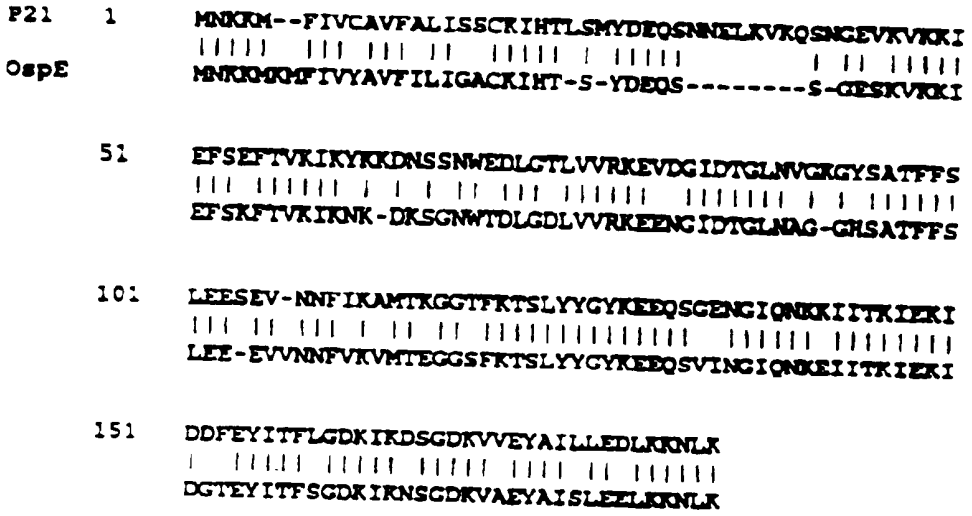
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Figure 7

	Sequence		
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ospB			AAGGAG
ospC	TTGAAA	TATAAA	AAAGGAGG
ospD	TTGATA	TATATT	AAGGAG
ospE	TTGTTA	TATATT	GGAG
ospF			AGGAG
cppA	TTAGTA	TATAAT	AGGAGA
p21	TTGTTA	TATATT	GGAG
k2			GGAG

ospA, *ospB*, *ospD*, and *eppA* are from *B. burgdorferi* strain B31; *ospC* is from *B. burgdorferi* strain pKo; *ospE*, *ospF*, *p21*, and *k2* are from *B. burgdorferi* strain N40.

Figure 8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/06610

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/31 C07K14/20 C12N15/62 C07K16/12 A61K39/02
G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INFECT IMMUN, AUG 1995, 63 (8) P3030-6, UNITED STATES, XP000615510 BRUNET LR ET AL: "Antibody response of the mouse reservoir of Borrelia burgdorferi in nature." see the whole document ---	1-30
X	EMBL Database entry BBORF; accession number L32797; 05 May 1995; XP002022265 SUK K ET AL.:"Borrelia burgdorferi genes selectively induced in the infected host" see sequence ---	2,4-30
X	EP 0 565 208 A (SYMBICOM AB) 13 October 1993 see page 5 - page 6 -----	9

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

3

Date of the actual completion of the international search

Date of mailing of the international search report

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer
Espen, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/US 96/06610

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Arucle 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 20,22,24
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 20,22,24 are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/ composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international applicauon, as follows:

see continuation-sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [X] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
subject 1. and subject 2. (see continuation-sheet)

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims, it is covered by claims Nos.:

[X] No protest accompanied the payment of additional search fees